NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
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Project Director

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Louisiana State University Medical Center
Neuroscience Center of Excellence

Volume 4 of 9

Neurochemical Protection of the Brain, Neural Plasticity and Repair

Project Director:
Nicolas G. Bazan, M.D., Ph.D.

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ANIMAL USE
SEPTEMBER 20, 1995 THROUGH SEPTEMBER 19, 1996

DAMD17-93-V-3013

The experimental animals used during this period for the project, Neural Responses to Injury: Prevention, Protection, and Repair, **Subproject: Neurochemical Protection of the Brain, Neural Plasticity and Repair**, are as follows:

Species	Number Allowed	Number Used	LSU IACUC#
Rat (Sprague-Dawley)	125	125	1046
Rat (Sprague-Dawley)	91	91	1045

Investigator Signature

Volume 4 Neurochemical Protection of the Brain, Neural Plasticity and Repair

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Bazan NG, Allan G, Marcheselli VL (1996) An inhibitor of injury-induced COX-2 transcriptional activation elicits neuroprotection in a brain damage model ***in*** Improved Non-Steroid Anti-Inflammatory Drugs: COX-2 Enzyme Inhibitors. Sir J Vane, J Botting and R Botting (eds), Kluwer Academic Publishers, Lancaster, United Kingdom, 9:145-166.

Marcheselli VL, Bazan NG (1996) Sustained Induction of Prostaglandin Endoperoxide Synthase-2 by Seizures in Hippocampus: Inhibition by a Platelet-Activating Factor Antagonist. J Biol Chem 271:24794-24799.

Kolko M, DeCoster MA, Rodriguez de Turco EB, Bazan NG (1996) Synergy by Secretory Phospholipase A_2 and Glutamate on Inducing Cell Death and Sustained Arachidonic Acid Metabolic Changes in Primary Cortical Neuronal Cultures. J Biol Chem. In press.

Bazan NG (1996) Lipid messengers and prostaglandin endoperoxide synthase-2 ***in*** neuronal cell death in Primers in Cerebrovascular Diseases. In press.

Bazan NG (1995) Synaptic Messengers, Inflammatory Mediators, and Neuronal Plasticity in Cerebral Ischemia. The Second International Workshop on Maturation Phenomenon in Cerebral Ischemia-Neuronal Recovery and Plasticity, December 1995, Tokyo, Japan. In press.

Bazan NG (1996) The inflammatory mediator platelet-activating factor and the inducible prostaglandin synthase (COX-2) gene in CNS diseases. Smith-Kline Beecham Pharmaceuticals Research Symposium on Inflammatory Cells and Mediators in CNS Diseases, 14-15 November, Collegeville, PA. In press.

Marcheselli VL, Bazan NG (1995) Inducible Prostaglandin Synthase and *ZIF*-268 mRNA Upregulation in Vasogenic Cerebral Edema: Inhibition by a PAF Antagonist. Soc Neurosci 21:1867, 1995.

Kolko M, DeCoster MA, Lambeau G, Lazdunski M, Bazan NG (1995) Effect of Secretory Phospholipases A_2 and Glutamate on Viability of Rat Cortical Neurons and Calcium Dynamics. Soc Neurosci 21:608, 1995.

DeCoster MA, Bazan HEP, Bazan NG (1995) Platelet-Activating Factor Induced Intracellular Calcium Oscillations in Rat Hippocampal Neurons. Soc Neurosci 21:1127, 1995.

Bazan NG (1995) PAF Modifies Cell Function and Pathology by Affecting Gene Expression. 5th International Congress on PAF and Related Lipid Mediators, 12-16 September 1995, Berlin, Germany.

Bazan NG, Cinar NH, Marcheselli VL (1996) Lipid second messengers and the upregulation of inducible prostaglandin synthase-2 triggered by vasogenic cerebral edema. The Joint Meeting of the American Society for Biochemistry and Molecular Biology, 2-6 June, New Orleans, LA.

Cinar H, DeCoster MA, Bazan NG (1996) Modulation of chronic neurotoxicity by combined kainate and glutamate treatment of hippocampal neurons. The Joint Meeting of the American Society for Biochemistry and Molecular Biology, 2-6 June, New Orleans, LA.

DeCoster MA, Bazan NG (1996) Platelet-activating factor modulates intracellular calcium dynamics in rat hippocampal neurons. The Joint Meeting of the American Society for Biochemistry and Molecular Biology, 2-6 June, New Orleans, LA.

Kolko M, Rodriguez de Turco EB, DeCoster MA, Bazan NG (1996) Modulation of calcium, neurotoxicity and arachidonic acid release by phospholipase A type II and glutamate *in vitro*. The Joint Meeting of the American Society for Biochemistry and Molecular Biology, 2-6 June, New Orleans, LA.

Bazan NG (1996) The inflammatory mediator platelet-activating factor and the inducible prostaglandin synthase (COX-2) gene in CNS diseases. Smith Kline Beecham Pharmaceuticals Research Symposium on Inflammatory Cells and Mediators in CNS Diseases, 14-15 November, Collegeville, PA.

THE KINDLING MODEL OF EPILEPTOGENESIS AND SYNAPTIC PLASTICITY

Introduction.

The development of chronic epilepsy is a very serious complication of head injury, neurodegenerative diseases, brain tumors, and exposure to neurotoxic agents. Head injury is often associated with loss of short-term memory, indicating trauma to the hippocampal formation, the brain region most commonly associated with epileptic brain damage. Underlying the formation of epilepsy (epileptogenesis) is proposed to be a vicious cycle initiated by the loss of neurons. In an attempt to repair and/or replace lost synaptic connections, the brain can develop aberrant synaptic circuits that permit the propagation and amplification of waves of excitatory neurotransmission, eventually resulting in prolonged or repeated seizures (status epilepticus). The massive amounts of excitatory amino acids released during these episodes can stimulate further neuronal loss (excitotoxic damage), the formation of more aberrant synaptic circuits, and further seizures (Choi and Rothman, 1990). Excitotoxic damage has been demonstrated in several experimental models of status epilepticus (Meldrum et al, 1973; Ben-Ari, 1995; Sloviter, 1987). Neuronal loss is most prominent in subfields of the hippocampus, particularly the CA1, CA3, and the hilus of the dentate gyrus; and in other limbic structures (Nadler and Cuthbertson, 1980; Ben-Ari et al, 1985), and closely matches the pattern of hippocampal sclerosis commonly seen in human temporal lobe epilepsy (Babb and Brown, 1987; Gloor, 1991).

It is now apparent, however, that even brief, sporadic seizures, such as those induced in the kindling model of epileptogenesis, may induce neuronal loss in vulnerable brain regions

(Cavazos et al, 1994). Kindling is a widely-used animal model of chronic epilepsy in which repetition of a given electrical stimulus to a discrete focal point in the limbic system of the brain gives rise to a fixed sequence of events. This sequence is characterized by a progression in both the lengths of the electrographic seizures (afterdischarges), and in the severity of the accompanying behavioral seizures until a stable plateau is reached. Subsequently, the same stimulus, which at the beginning of the process elicited a minimal afterdischarge, will then elicit a protracted afterdischarge and stereotyped generalized convulsions characteristic of kindled seizures. (Goddard et al, 1969; McNamara et al, 1985; Racine and Burnham, 1984). Kindled seizures can then subsequently be elicited in treated animals even when the stimulus is witheld for several months, which establishes that chronic alterations have occurred in the kindled brain analogous to those in the epileptic human brain. Hippocampal kindling has been shown to induce collateral sprouting of mossy fibers (Messenheimer et al, 1979; Geinisman et al, 1988), and progressive loss of interneurons in the hilus of the dentate gyrus, and in the CA1 (Cavazos and Sutula, 1990; Cavazos et al, 1994).

Based on the observations of Goddard et al, (1969) many kindling protocols have usually employed once-daily stimuli of about 1 second duration, with about 20-30 treatments required to reach a stable plateau of seizure intensity. There is a refractory period of at least 90 minutes before a second stimulus of this type will elicit a kindled seizure (Mucha and Pinel, 1977). More recently, it has been found, however, that increasing the duration of the stimulus to around 10 seconds overcomes this refractory period, thus allowing a series of closely-spaced stimuli to elicit kindling responses over a few days (rapid kindling, Lothman et al, 1988). Additionally, whereas carrying out the rapid kindling protocol on successive days (12 stimuli at 30 minute intervals/day) rapidly elicits a state resembling kindling, but which decays in a few days, an alternating day protocol of stimulus-rest in which the same total number of stimuli are delivered for a permanantly kindled state (Lothman and Williamson, 1994). Non-permanent kindling-type responses can in fact be seen in as little as 6-8 hours, which implies that there are distinct mechanisms involved in this form of epileptogenesis; a relatively rapid, stimulus-evoked increase in brain excitability, and a slower underlying process in which a permanantly epileptic state is established.

This laboratory has been interested in the roles of platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) in epilepsy and other forms of neurotrauma. PAF is a bioactive, membrane phospholipid-derived messenger involved in many inflammatory and immune responses (Braquet et al, 1988; Prescott et al, 1990). A cell-surface receptor of the rhodopsin-type superfamily has been cloned and characterized (Honda et al, 1991), and in addition, a second, intracellular binding site has been found in cortical and hippocampal microsomes (Marcheselli et al, 1989; Marcheselli and Bazan, 1994). We have shown that PAF has short-term effects on glutamate release (Clark et al, 1992) and long-term potentiation (Kato et al, 1993) mediated through presynaptic receptors, and on seizure-induced gene expression mediated through the intracellular receptor (Marcheselli and Bazan, 1994, 1996). The intracellular receptor is specifically sensitive to the hetrazepine PAF antagonist BN 50730 (Marcheselli and Bazan, 1994). We have shown that pretreatment of animals with BN 50730 blocks induction of the inducible form of prostaglandin synthase (COX 2) during seizures induced by a single electroconvulsive shock (ECS) and intraperitoneal injection with kainic acid (KA) (Marcheselli and Bazan, 1996). COX 2 induction is much greater in the KA model, in which neuronal death is induced, than in the ECS model, which does not induce neuronal damage. We have thus been exploring the idea that under pathophysiological conditions, PAF is a messenger in neuronal apoptosis, perhaps acting through induction of COX 2, and therefore that drugs directed at PAF activity will be protective against excitotoxic neuronal death. This current report contains our findings on the effect of BN 50730 on neuronal loss during kindling, and whether this is the mechanism by which the drug slows the progression of kindling.

BODY

Previous work.

We have set up a method for rapid hippocampal kindling of rats, using a computer-controlled apparatus to automatically deliver the stimuli and record electrographic responses. This protocol has two major advantages over traditional kindling protocols: 1. The time required to generate fully-kindled animals is much less (electrode implantation, 7 days' recovery, 25-30 once-daily stimuli followed by 7 days before re-testing = 39-44 days versus 7 + 7 + 7 = 21 days for the rapid method); 2. The computer-controlled apparatus makes stimulus delivery and data collection much less labor-intensive. **Year 1** was devoted to constructing the apparatus and troubleshooting the protocol. A emthod was devised to deliver drugs during the kindling period using implanted cannulae attached to mini osmotic pumps. During **year 2**, the rapid kindling model was used to assess the effects of the PAF antagonist BN 50730 on the development of electrographic and behavioral seizures. Drug-treated animals showed a statistically-significant lower increase in evoked after-discharge duration (ADD), and a slower development of behavioral seizures.. Preliminary histological studies suggested that BN 50730 was protective against cell loss in the hilus of the dentate gyrus.

Objectives, year 3

The major objective during year 3 was to establish at which part of the kindling mechanism BN 50730 was acting to slow down the process. Preliminary histological analysis had suggested that BN 50730-treated animals showed reduced hilar cell loss following the kindling procedure. Our aims were to establish if some of this cell loss was through apoptosis, and if BN

50730 acted by inhibiting the apoptotic mechanism.

MATERIALS AND METHODS

Kindling

Electrode and mini-pump placement.

Animals (male Sprague-Dawley rats 250-275g) were anesthetized (ketamine/xylazine i.p. with halothane supplement if animals showed signs of arousal), shaved in the scalp area an positioned in a stereotaxic apparatus. The scalp was excised to expose the skull and a hole drilled with a 0.04" diameter dental drill fitted with a 1.0mm depth drill stopper. A platinum tripolar stimulating/recording electrode was placed stereotaxically in the right ventral hippocampus (coordinates relative to bregma: 0.50 ventral, 0.49 lateral, 0.36 posterior) with the tips near the inner blade of the dentate gyrus (Figure 1). A brain infusion kit (Azlet) was implanted at the same time. It consisted of a mini osmotic pump loaded with artificial cerebro-spinal fluid (CSF) implanted under the skin in the scapular region, an interconnecting Teflon micro-catheter with rubber connectors at each terminal, also implanted under the skin, and a cannula stereotaxically implanted in the cerebral ventricle (coordinates relative to bregma: 0.03 ventral, 0.25 lateral, 0.08 posterior). The skin was sutured closed with only the electrode's pin connector exposed. The connector was secured in place with two screws (one laterel to the central fissure and the other in the temporal bone) and dental cement.

Kindling.

After electrode and mini pump placement, rats were allowed to recover for a period of 6 days. 24 hours before stimulation, animals were anesthetized with ether, and the Teflon microcatheter replaced with a new microcather containing either fresh CSF, BN 50730 (10mg/kg dissolved in DMSO at 3μg/μl), or vehicle (DMSO alone). Animals infused with CSF, but not stimulated, were used as controls to assess the effects of the kindling process on non drug-treated animals. DMSO-infused, stimulated animals were used to control for the effects of the vehicle in drug-treated, stimulated animals. The next day, the animals' afterdischarge threshold (ADT) was determined. The ADT is the minimum stimulus required to produce an electrographic discharge, and for animals with correct electrode placement should be in the range 40μA-70μA. Animals were then stimulated every 30 min for a 6 h period with a 400 μA signal, presented at a frequency of 50 Hz for 10 seconds. Following a repolarization period of 100 mS, EEGs were recorded for up to 2 min. Stimulation occurred throughout days 1, 3, 5, and 7 (Figure 2). The stimulation/recording apparatus is described in detail in the Year 1 report on this project. Essentially, it consists of an 8-channel computer-controlled stimulator/recorder. Cables running from the stimulation apparatus are attached to the electrode assembly on the head of each animal using a swivel device, allowing free movement. Each animal was kept in an open lucite box with free access to food and water for the duration of the stimulation process, then returned to their cages. Animals were stimulated in series, and the electrographic data of the stimulus and EEG response digitized and stored on video tape for later analysis. Behavioral seizures were scored by the operator using the scale of Racine (1972), where class 1 = facial clonus, class 2 = head nodding and "wet dog" shaking, class 3 = forelimb clonus, class 4= rearing sitting and standing, class 5 = rearing and falling.

Animals were then allowed a 7-day "development" phase during which no stimuli were delivered. After this, animals were subjected to 2 stimuli/day (same parameters as before) over a 14-day period to test their retention of kindled responses. The kindling protocol is outlined in

Figure 2.

General histological procedures.

Rats were killed at days 0, 14, 16, 21, and 28 of the kindling protocol (times A, B, C, D, and E in Figure 2). Brains were removed and halved, and then each hemisphere cut at an angle to yield two hippocampal halves. These were placed in 4% formaldehyde in PBS at 4° C for 4 days. They were then dehydrated through an ethanol series to xylene, and embedded in paraffin. 10 μm-thick cross sections were cut from the central hippocampus and mounted on subbed slides. These were subsequently stained for a general histological appraisal with cresyl violet to favor Nissl substance (RNA) and nuclei (Humanson 1972). Histological evaluation of DNA fragmentation and apototic cell death was achieved by analysis of TUNEL stained sections using an Apoptosis Detection Kit, fluorescein (Promega, Madison, WI) according to the manufacturer's instructions. Propidium iodide (Sigma, St Louis, MO) was used as a counter stain for all other nuclei.

RESULTS

Seizure description: afterdischarge duration and behavioral seizure score.

The afterdischarge duration (ADD) is defined as the time during which the post-stimulus burst of electroencephalographic activity remains greater than three times background. ADD were plotted as a function of stimulation number during the acquisition phase of the kindling protocol (Figure 3). The ADD values for control animals increased markedly with successive stimulations, lasting as long as 145 seconds in some animals by the final day of stimulus. However, the BN-50730 treated animals maintained ADD values of only about 60 seconds, significantly lower than control animals ($p < 0.0001$ using two level factorial ANOVA, followed by t-tests on least square means). Interestingly, ADDs were not significantly different between drug-treated and control groups after the first day, but diverged later during the process.

Behavioral seizure scores were also graphed for each of these animals (Figure 4). Class 5 seizures were achieved usually near the end of the third stimulation period (day 5) and were, again, typically observed throughout the latter half of day 7. Generally, the degree of seizure was reduced by one stage (from stage 5 to stage 4) in BN-50730 treated animals.

Histological analysis and evaluation of cell bodies.

Cresyl violet stained sections revealed hippocampal, hilal, and dentate gyral cell bodies to be rounded with clear cytoplasm and slightly contrasted nuclei. This was apparent in samples from control rats (Figure 2, time point A). General survey sections (Figure 5) revealed normal hippocampal structures. Five regions of interest were selected, and are shown at higher magnification (Figures 6-10). All cell bodies appear normal.

Similar survey sections were taken after 14 days of the kindling protocol (Figure 2, time point B). These cresyl violet stained sections revealed the beginning of cell compromise within specific regions (Figure 11). The same 5 regions of interest were selected and viewed at higher magnification (Figures 12-16). Region 1, just peripheral to the hippocampal CA1 cells, showed darkened, reduced, highly angled cell bodies among normal CA1 cell bodies (Figure 12), while the CA1 fibers generally showed no compromise (Region 2; Figure 13). Also, the CA3 cells appeared normal (Region 3; Figure 14). Changes again began to appear at the very end of the CA3 cell array, accompanied by darkened cells within the hilar region (Region 4; Figure 15). Finally, some of the inner cell bodies of the Dentate gyrus revealed darkening (Region 5; Figure 16). It is important to note here than no cells appeared to have been lost, although many had become reduced in size.

To determine if apoptotic cell death was occurring at this time, these same 5 regions of interest were examined on nearby sections from these same blocks by TUNEL labeling (Figures 17-21). Several nuclei fluoresced, indicating an extremely low level of apoptotic cell death. One CA1 nucleus is indicated in Figure 17, but none in adjacent regions (Figure 18). In the CA3 region, two nuclei are visible (Figure 19), and one on the inner edge of the dentate gyrus (Figure 20). No label was found along the inner blade (Figure 21).

General survey sections were also taken at 16 days into the kindling protocol (Time point C; Figure 2), two days into the retest phase. These cresyl violet sections showed extensive cell damage within all 5 regions of interest (Figures 23-27). Figure 23 demonstrates extensive cell darkening near the origin of the CA1 cell bodies, but reveals many normal CA1 cells as well. Although region 2 contains darkened, shrunken CA1 cells exclusively, cell counts show little or no cell drop out (Figure 24). Region 3 is similar (Figure 25); the CA3 cells are dramatically affected. Some healthy cells are apparent at the end of the CA3 column and within the hilas, but most cells appear darkened (Figure 26). Approximately 50% of the cells of the inner blade of the dentate gyrus are also affected, exhibiting dark, shrunken profiles (Figure 27).

TUNEL stained sections of regions 1-5 show no cell death occurring by apoptosis. Typical sections, within which there is no label, are shown in Figures 28-30.

Finally, survey sections were obtained for the midpoint and the endpoint of the retest phase (Time points D and E; Figure 2). While these showed a slight increase in darkening cells throughout the hippocampus, no significant differences between these time points and the 16 day, time point C, were observed. Additional sections reacted for TUNEL labeled DNA did not demonstrate any increase in apoptotic nuclei, and general cell counts suggest that at these times cell loss is minimal.

Figure 31 demonstrates a positive control for TUNEL labeling of rat hippocampal apoptotic nuclei. In this case, kainic acid was injected i.p. After 2 weeks, animals were killed and hippocampal sections were reacted for TUNEL, following the same protocol as used above on kindled hippocampal sections. This one example demonstrates massive labeling of apoptotic nuclei within the dentate gyrus. While all nuclei show a positive TUNEL reaction, indicating that the kianic acid had affected these cells over the previous two week interval, the most recent nuclear damage had occurred to the inner-most layer of dentate cells. These appear as a single bright row of yellow nuclei. This study, this positive control, demonstrates the effectiveness of the TUNEL labeling technique in rat hippocampal tissue.

CONCLUSIONS

ADD values increased throughout the acquisition phase of the kindling protocol in control-stimulated animals, but these increases were repressed in the presence of BN-50730. Behavioral seizure scores increased in control-stimulated animals, to finally result in consistent class 5 seizures, but BN-50730 treated, stimulated animals achieved only class 4 seizures. Interestingly, ADDs were not significantly different between drug-treated and control groups after the first day, but diverged later during the process. This indicated that BN 50730 was not acting as an anticonvulsant, but was inhibiting some process underlying the development of kindled seizures. Even so, given that the BN 50730-treated animals still developed class 4 seizures, repressing the kindling-induced ADD increase was not in itself sufficient to fully block the development of kindled behavioral seizures.

In the hippocampi of unstimulated animals (unoperated or electrode implanted but not stimulated animals), CA1, CA2, and CA3 cell bodies, and those of the dentate gyrus and hilar regions, appeared round. In tissues from stimulated animals throughout the retest phase, many of the cell bodies within these regions appeared to be smaller, darkened, and angular in shape. The number of cell bodies undergoing these changes increased with the number of post-kindling stimuli.

The number of cell bodies within the areas of the hippocampus, dentate gyrus, and hilus remains constant throughout the retest phase; there is no noticable cell death occurring. When hippocampal sections from these same blocks are examined for apoptotic cell death by TUNEL, only occasional apoptotic figures were seen; no apoptosis occurred throughout the retest phase.

Thus, while some initial cell death occurs immediately within the acquisition phase, many nuclei survive throughout the development and retest phases. The large drop in hippocampal, dentate gyral, and hilar cells must, therefore, occur as a gradual process as a result of continual seizure episodes following the kindling process. Therefore, neural sprouting and rewiring, known to occur early in the kindling process, are either an independent process, separate from that producing apoptosis, or are a continual process that accompanies a slow, low level apoptotic process.

It should be pointed out, that although neuronal damage during the kindling process is quantitatively minor compared to other epileptogenic treatments, such as kainic acid administration (Ben-Ari, 1985, and see Figure 31), the outcome of this damage in terms of the development of a state corresponding to human chronic epilepsy is clearly very severe.

FURTHER STUDIES

- Continue histological studies to assess the effects of BN 50730 on hippocampal neuron loss an new sprouting during the kindling process.

- To determine the stage(s) in the kindling process in which PAF may be involved:

  - Study expression of immediate early gene expression (protooncogenes, COX 2), quantitatively using Northerns, quantitative RT-PCR and Westerns, qualitatively using histological techniques.

  - Monitor the stimulation of rapidly responding nuclear transcription factors using electrophoretic mobility shift assays.

  -Assess changes in the activation of protein kinase signal transduction cascades.

REFERENCES

Babb TL, Pretorius JK, Mello LE, Mathern GW, Levesque MF 1992 Synaptic reorganizations in epileptic human and rat kainate hippocampus may contribute to feedback and feedforeward excitation. In: Molecular Neurobiology of Epilepsy. Engel J, Wasterlain C, Cavalheiro EA, Heinemann U, Avanzini G editors. Elsevier, Amsterdam, pp 193-204.

Charriaut-Marlangue C, Margaill I, Represa A, Popovici T, Plotkine M, Ben Ari Y 1996 Apoptosis and necrosis after reversible focal ischemia: An *in situ* DNA fragmentation analysis. J Cereb Blood Flow Metab 16:186-194.

Humason G 1972 Animal Tissue Techniques. Third Edition. WHFreeman, SanFrancisco, pp 219-220.

Kraus JE,Yeh G-C, Bonhaus DW, Nadler JV, McNamara JO 1994 Kindling induces the long-lasting expression of a novel population of NMDA receptors in hippocampal region CA3. J Neurosci 14:4196-4205.

Lothman EW, Williamson JM 1994 Closely spaced recurrent hippocampal seizures elicit two types of heightened epileptogenesis: A rapidly developing, transient kindling and a slowly developing, enduring kindling. Brain Res 649:71-84.

McNamara JO, Morrisett R, Nadler JV 1992 Recent advances in understanding mechanisms of the kindling model. Adv Neurol 57:555-560.

Pollard H, Cantagrel S, Charriaut-Marlangue C, Moreau J, Ben Ari Y 1994a Apoptosis associated DNA fragmentation in epileptic brain damage. Neuroreport 5:1053-1055.

Pollard H, Charriaut-Marlangue C, Cantagrel S, Represa A, Robain O, Moreau J, Ben-Ari Y 1994b Kainate-induced apoptotic cell death in hippocampal neurons. Neuroscience 63:7-18.

Represa A, Niquet J, Pollard H, Ben-Ari Y 1995 Cell death, gliosis, and synaptic remodeling in the hippocampus of epileptic rats. J Neurobiol 26:413-425.

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Figure 18
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Figure 19
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Figure 22
Light micrograph of control hippocampus, time point C

Figure 23
High magnification of area 1, time point C

Figure 24
High magnification of area 2, time point C

Figure 25
High magnification of area 3, time point C

Figure 26
High magnification of area 4, time point C

Figure 27
High magnification of area 5, time point C

Figure 28
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Figure 29
High magnification of area 3, time point C, stained for apoptotic nuclei

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Figure 31
Control hippocampal section, kianic acid triggered cell damage, stained for apoptotic nuclei

Figure 1
Electrode placement within right hippocampus. All electrode tips were placed near the inner blade of the dentat gyrus, below the CA1 region of the hippocampus. These electrodes were used as both stimulating and recording devices. Magnification 20x at microscope.

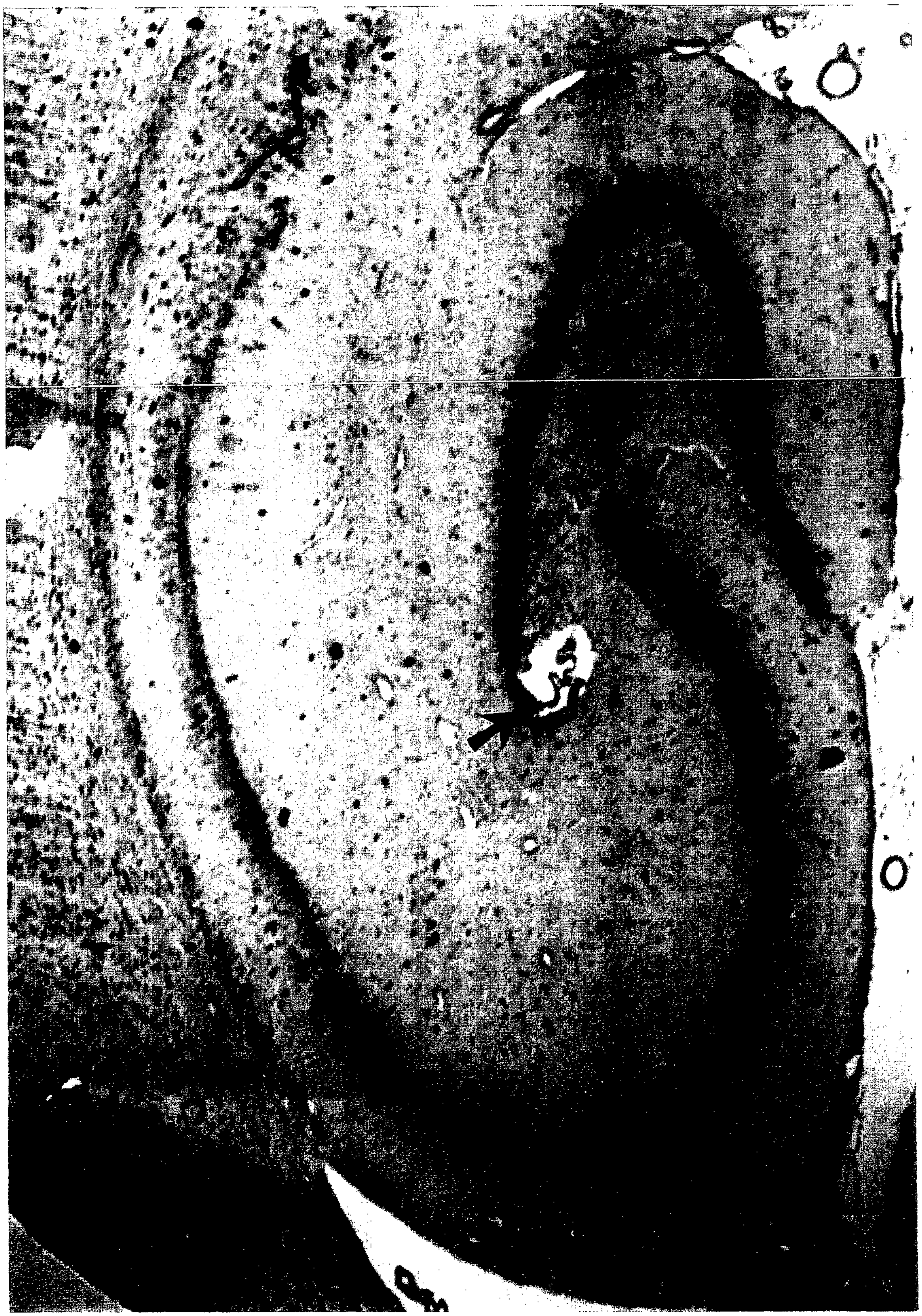
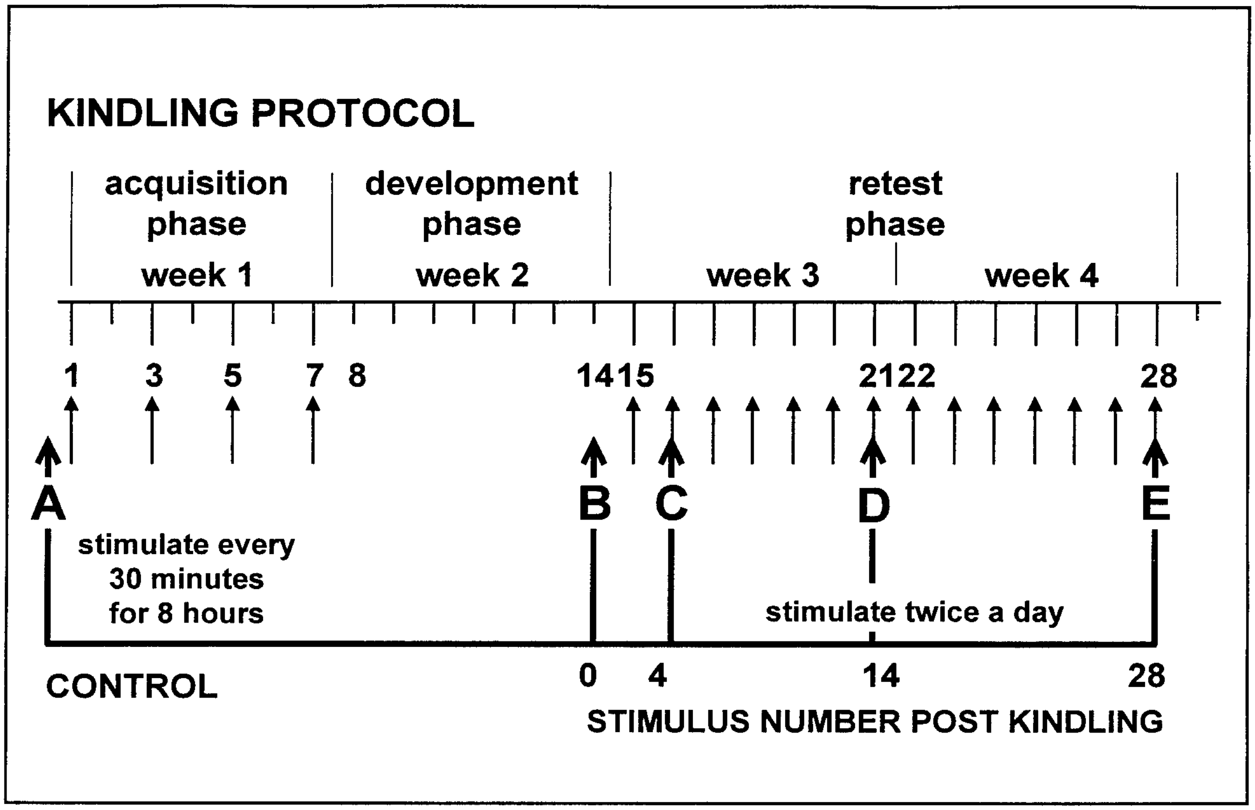


Figure 2

Kindling protocol, stimulus points, and sample times. Following electrode implantation, animals were allowed to recover for 1 week. They were then stimulated electrically at a subthreshold intensity once each 30 min for 8 h on days 1, 3, 5, and 7 to induce kindling. The second week, the development phase, animals were allowed to rest. From days 14 through 28, animals were stimulated twice each day and the electrical activity recorded. All stimulation time points are indicated by the small arrows; all tissue sampling points are indicated by the large arrows and the letters A, B, C, D, or E. Animals were given 0, 4, 14, or 28 post-kindle stimuli (the lowest axis).



KINDLING PROTOCOL

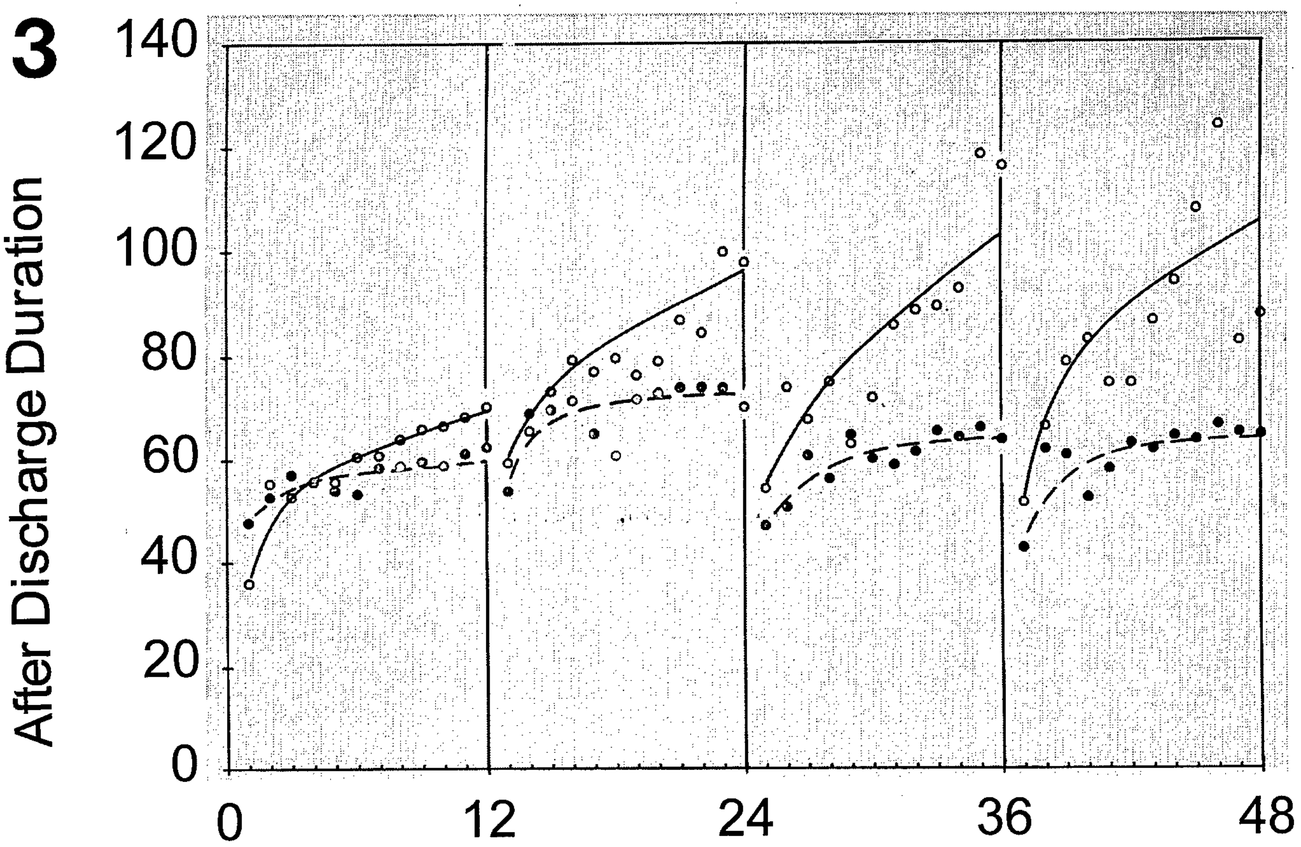
acquisition phase
development phase
retest phase
week 1
week 2
week 3
week 4
1
3
5
7
8
14
15
21
22
28
A
B
C
D
E
stimulate every 30 minutes for 8 hours
stimulate twice a day
CONTROL
0
4
14
28
STIMULUS NUMBER POST KINDLING

Figure 3

After-discharge duration. The after discharge duration is defined as the time during which the post-seizure burst of activity remains greater than three times background. The stimulus is set to just produce an after discharge and is not altered throughout the kindling process. Normally, this is about 40 μA. Durations were measured in seconds. The solid lines, open circles are from normally kindled rats. The dashed lines, closed circles are from BN-50730 treated rats. Each panel represents one day and corresponds to day 1, 3, 5, or 7 of the first week (the acquisition phase). This plot demonstrates that BN-50730 treatment greatly reduces the effects of the stimulation on the electrical patterns of the EEG. Each point represents the average of 8 animals.

Figure 4
Behavioral seizure scores. The animals described in figure 3 were also ranked by degree of seizure (stage 0 - 5) according to Racine's seizure classification. Generally, the degree of seizure was also reduced with BN-50730 treatment.

3


After Discharge Duration
0
20
40
60
80
100
120
140
0
12
24
36
48

4

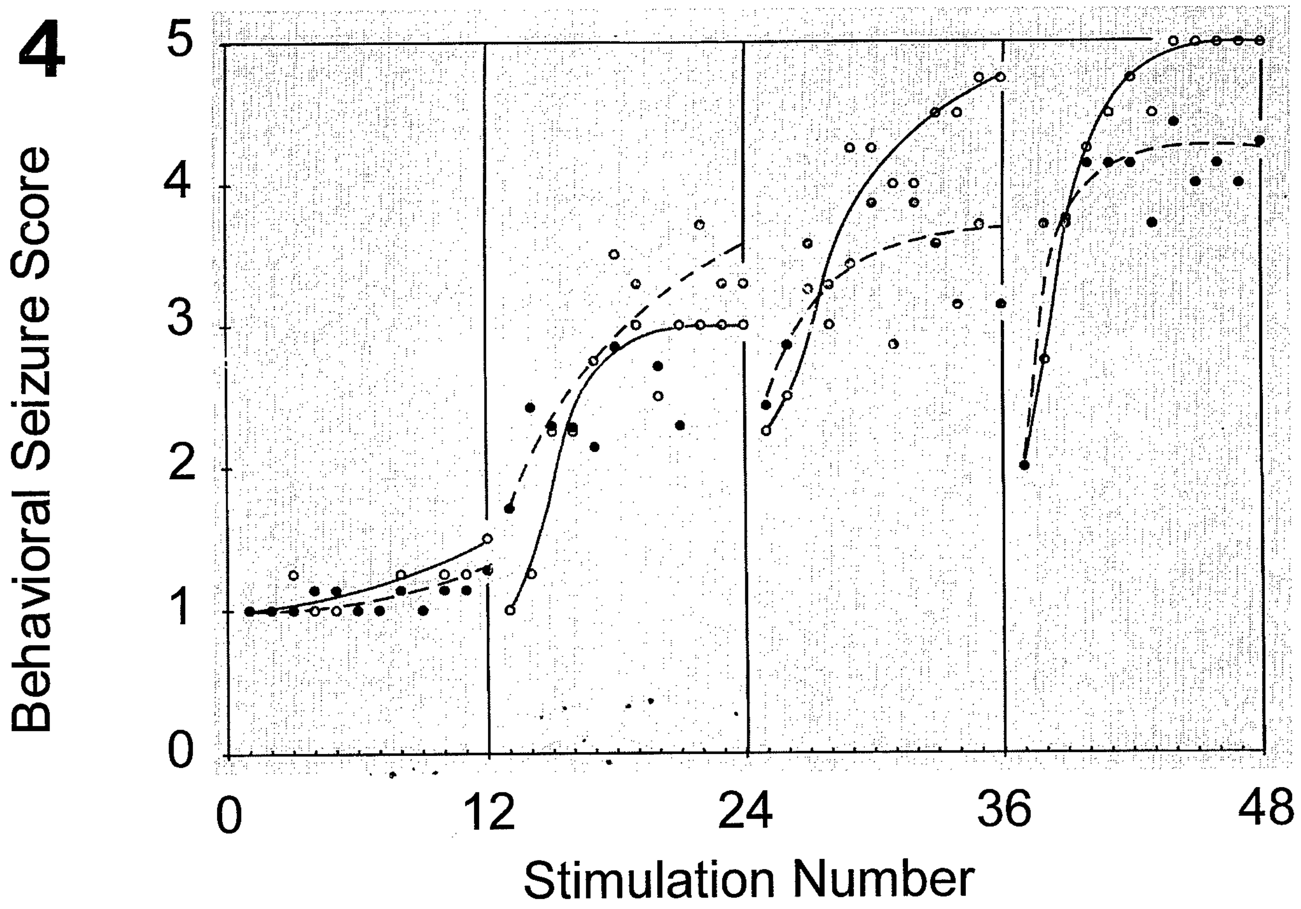

Behavioral Seizure Score
0
1
2
3
4
5
0
12
24
36
48
Stimulation Number

Figure 5

Light micrograph of control hippocampus, time point A. This control animal shows a normal hippocampus with no abnormalities. Cell numbers are normal and their appearance typical. Five regions have been selected for detailed analysis. Region 1 is just outside the hippocampus; region 2 represents the CA1 cells; region 3 represents the CA3 cells; region 4 represents the hilus; region 5 represents the inner blade of the dentate gyrus. Microscope magnification, 20x.

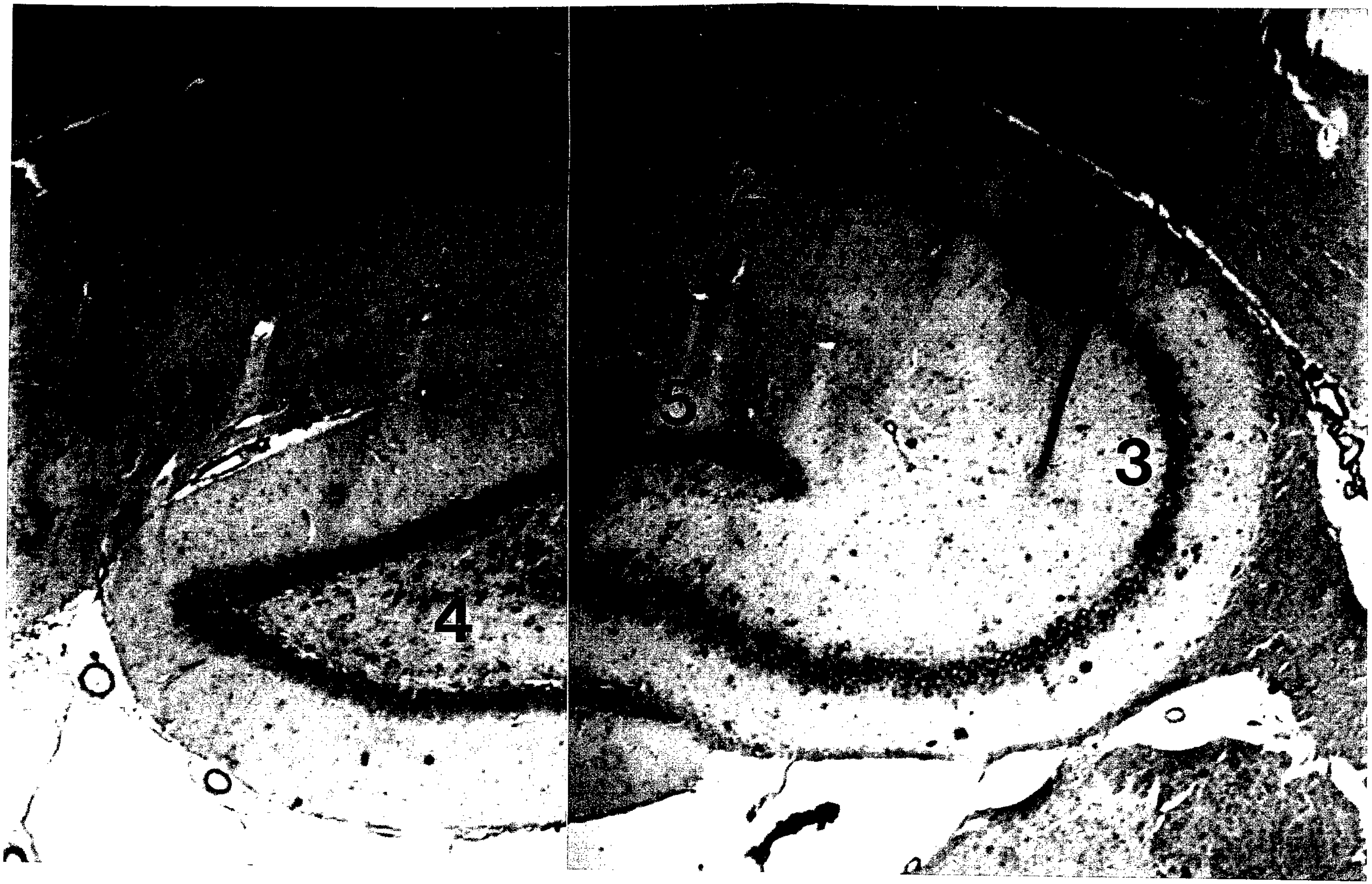

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Figure 6
High magnification of area 1, time point A. Microscope magnification, 100x.

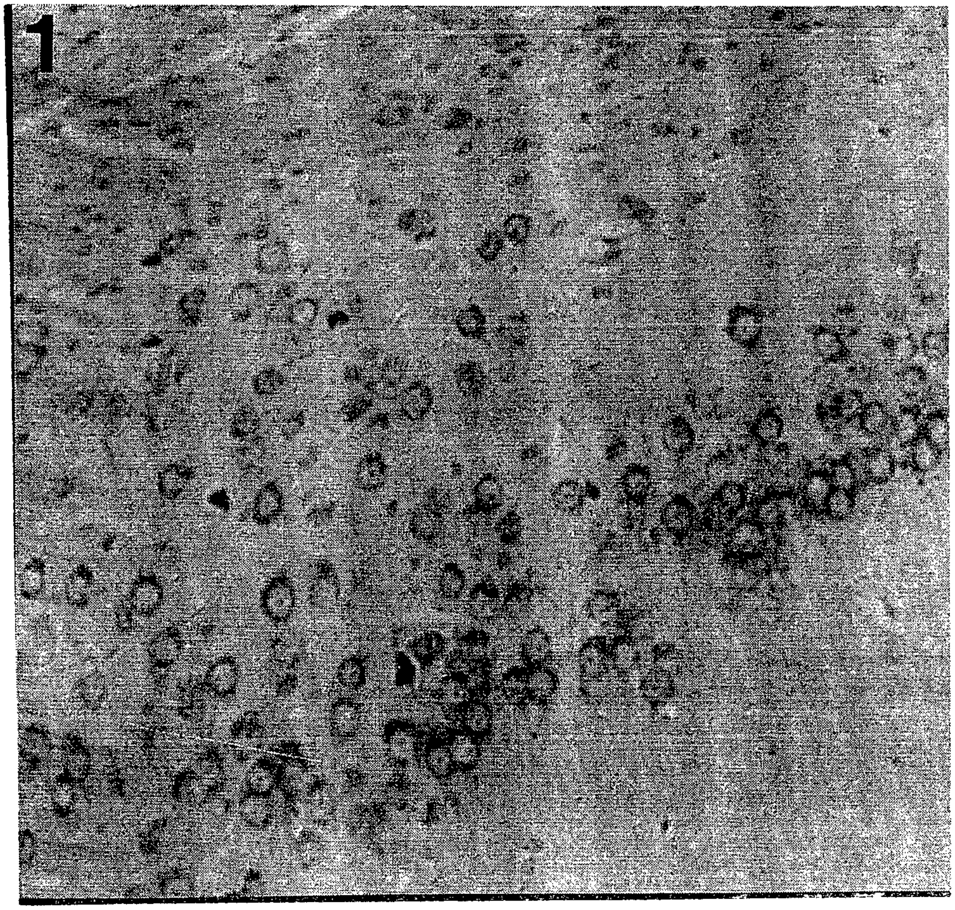
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Figure 7
High magnification of area 2, time point A. Microscope magnification, 100x.

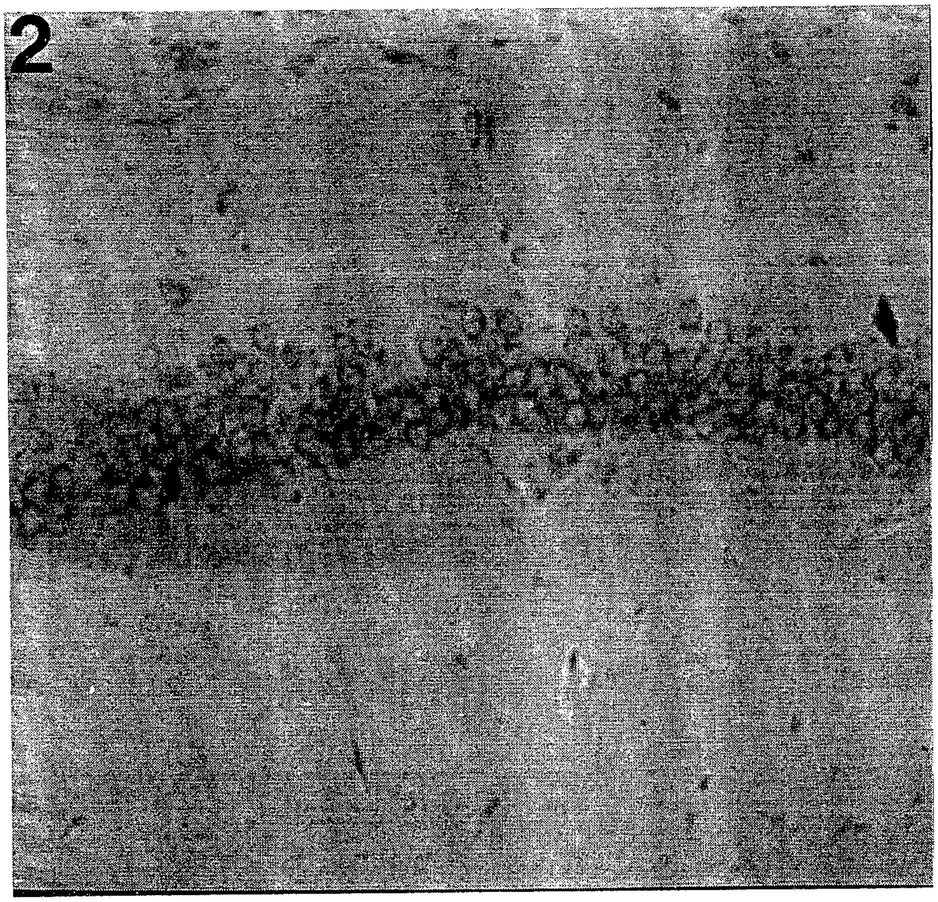
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Figure 8
High magnification of area 3, time point A. Microscope magnification, 100x.

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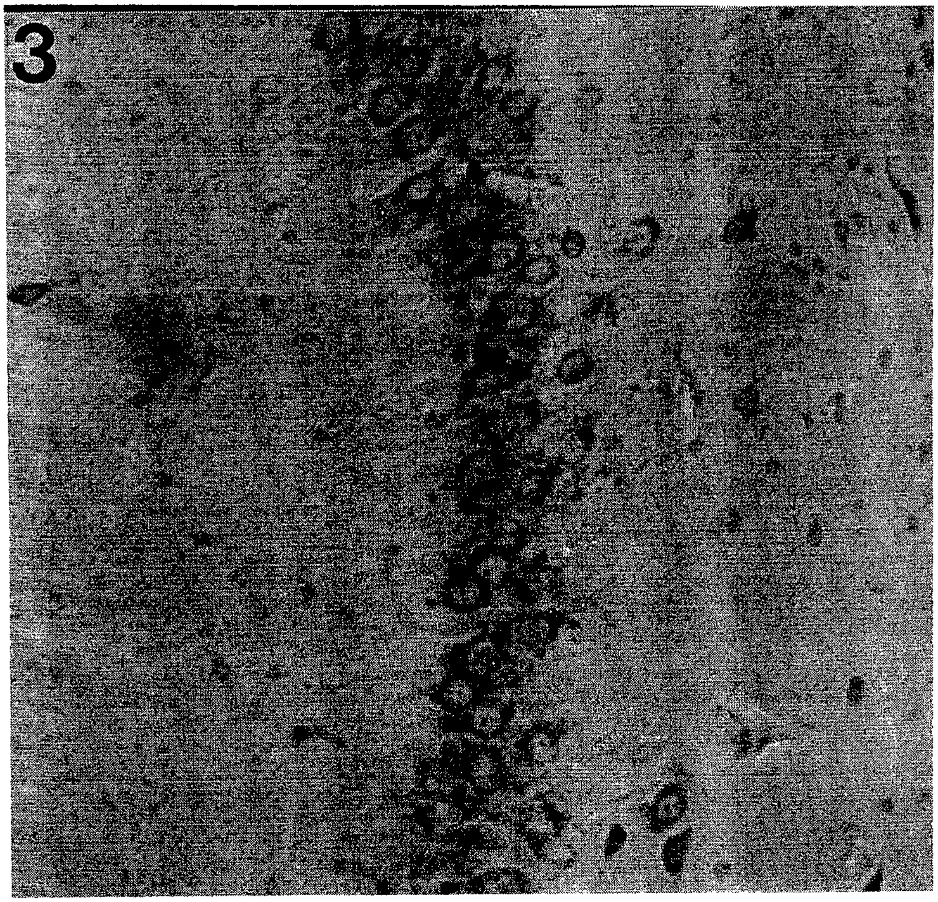

Figure 9
High magnification of area 4, time point A. Microscope magnification, 100x.

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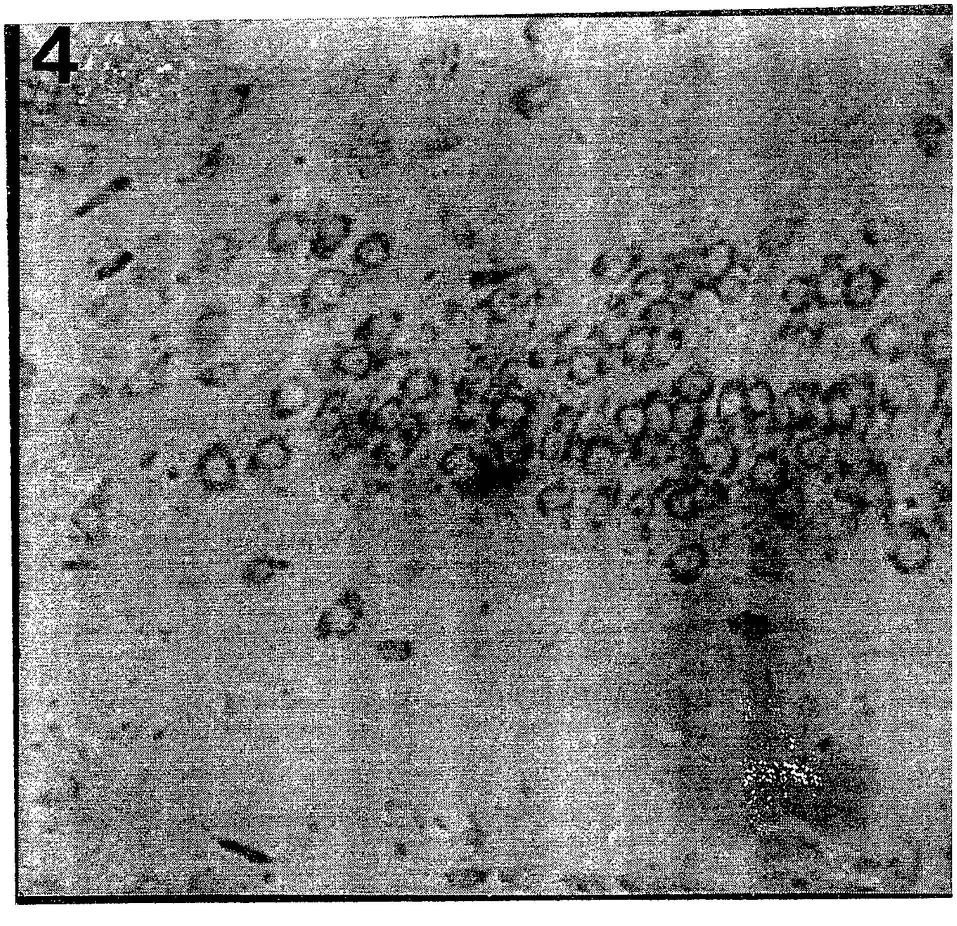

Figure 10
High magnification of area 5, time point A. Microscope magnification, 100x.

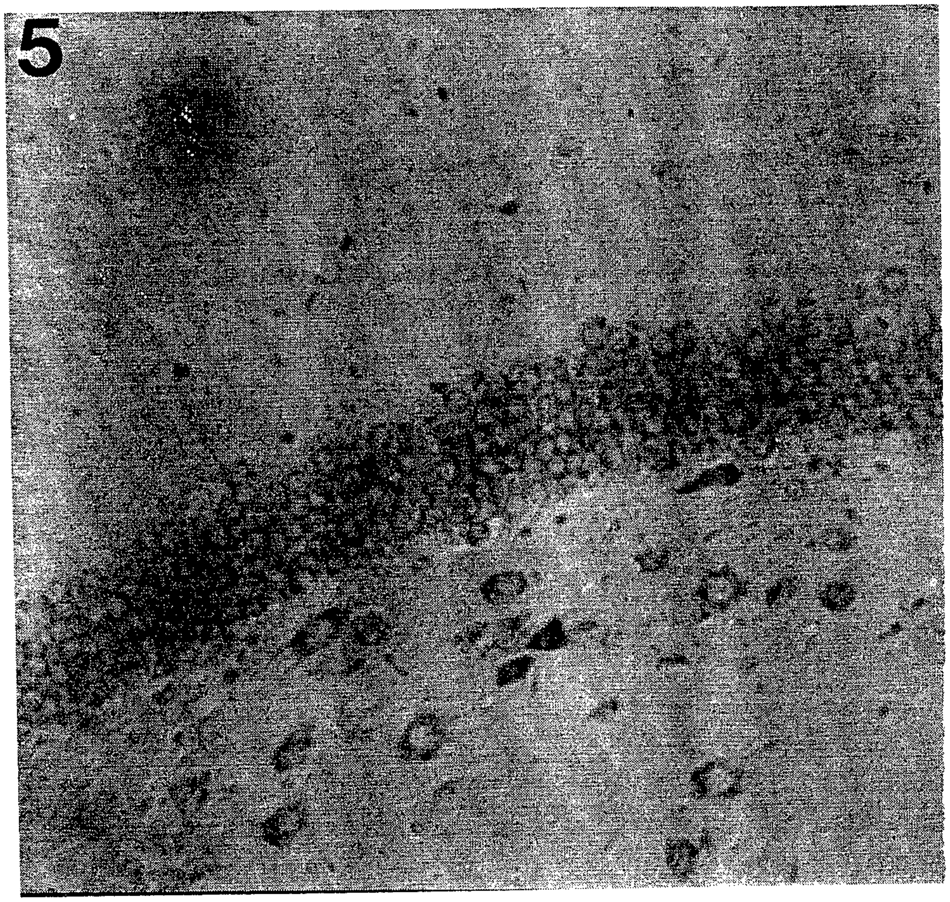

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Figure 11
Light micrograph of control hippocampus, time point B. This section demonstrates the hippocampus following kindling but with no subsequent stimulations. Some regions are beginning to show alterations. These include the CA1 cells near area 2, the CA3 cells near area 4, and the cells near the ends of the dentate gyrus. Microscope magnification, 20x.

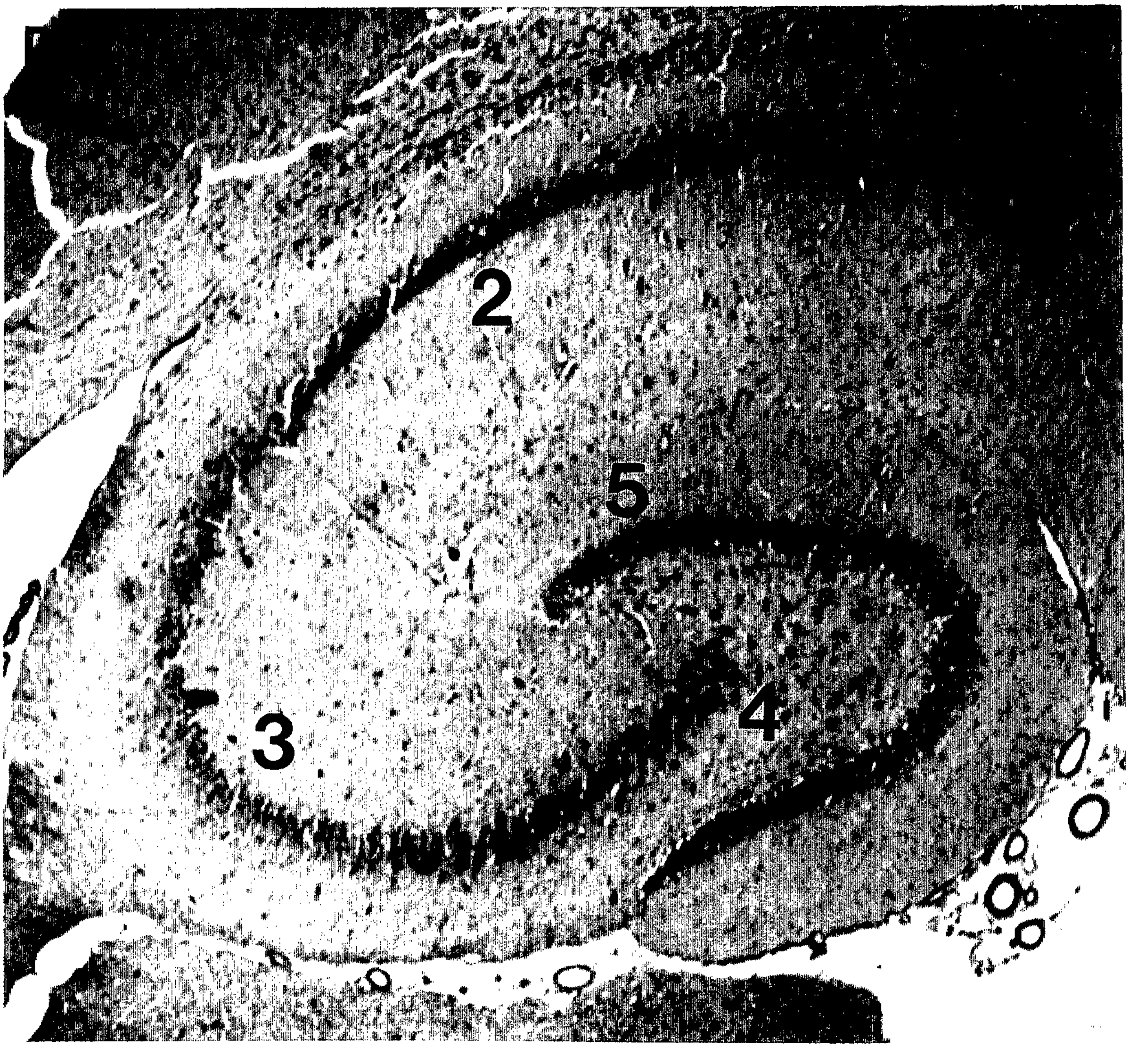
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Figure 12
High magnification of area 1, time point B. Notice some normal cell bodies with rounded appearance, and some now beginning to shrink, darken, and become angular. Microscope magnification, 100x.

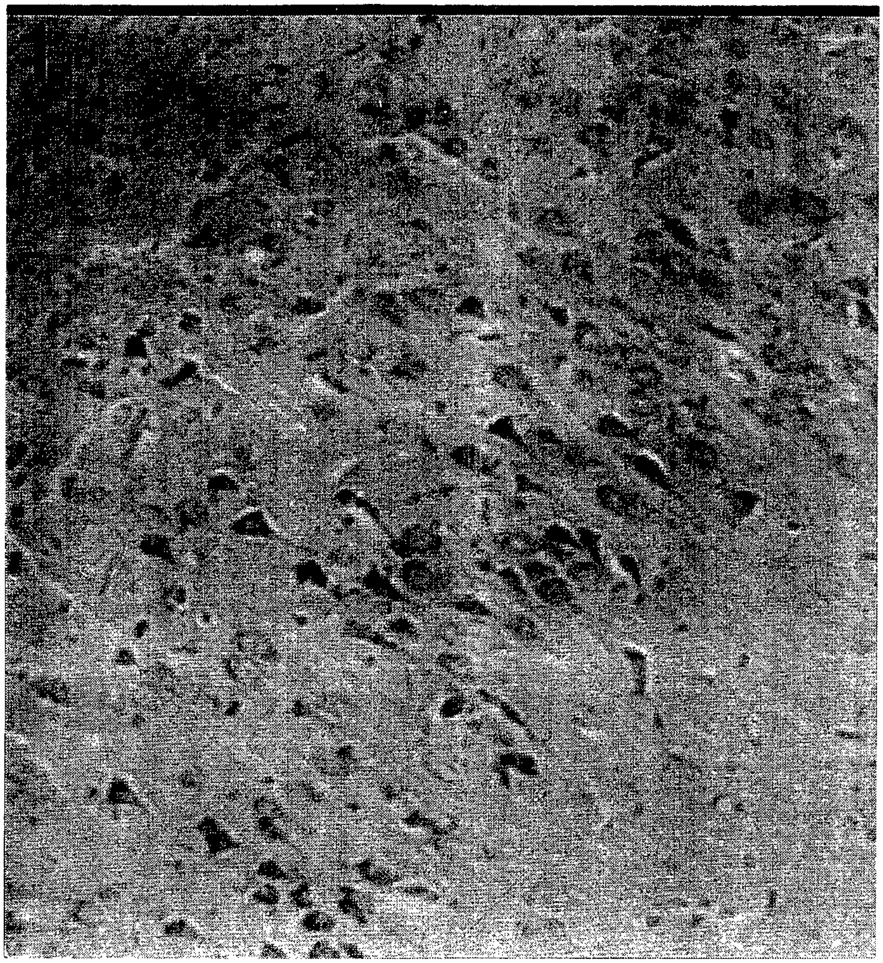

Figure 13
High magnification of area 2, time point B. Notice a few cells now darkening at the right of the photograph. Microscope magnification, 100x.

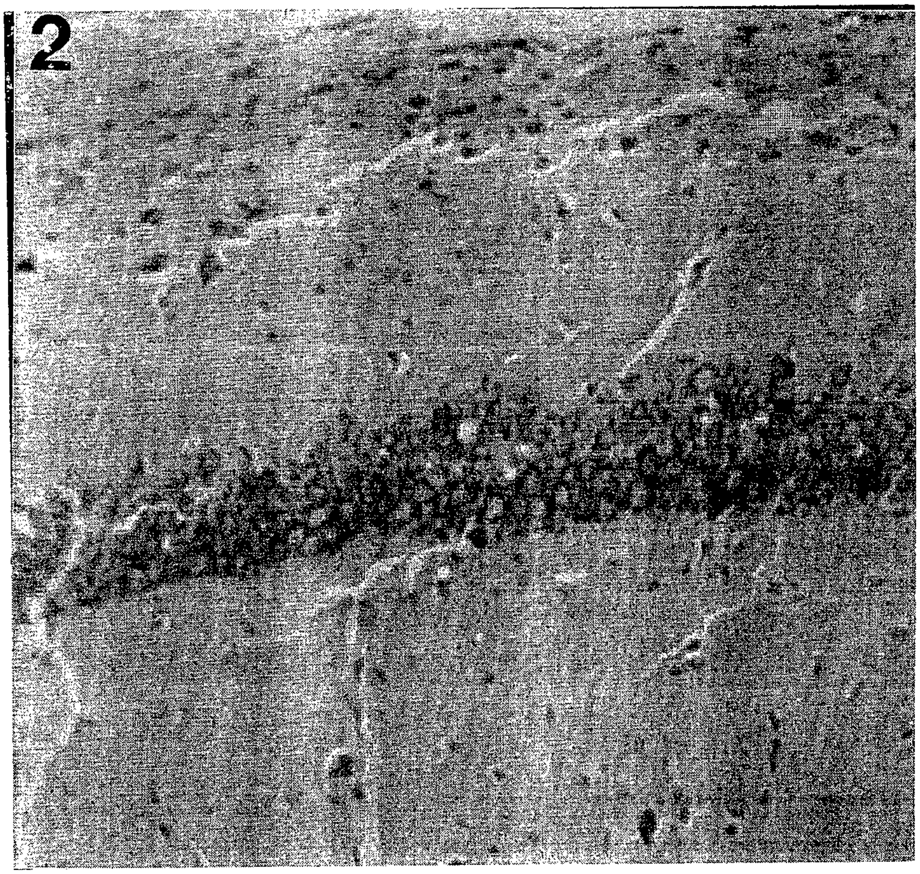
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Figure 14
High magnification of area 3, time point B. These cell bodies appear normal. Microscope magnification, 100x.

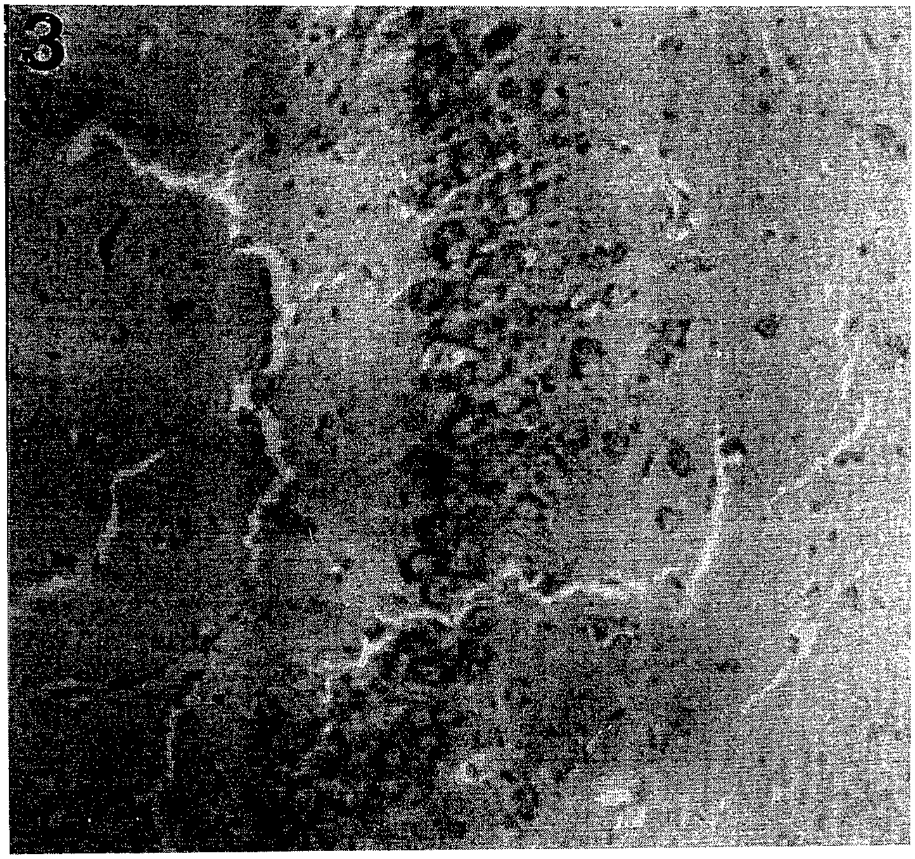
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Figure 15
High magnification of area 4, time point B. Notice that these cells are becoming dark and angular. Microscope magnification, 100x.

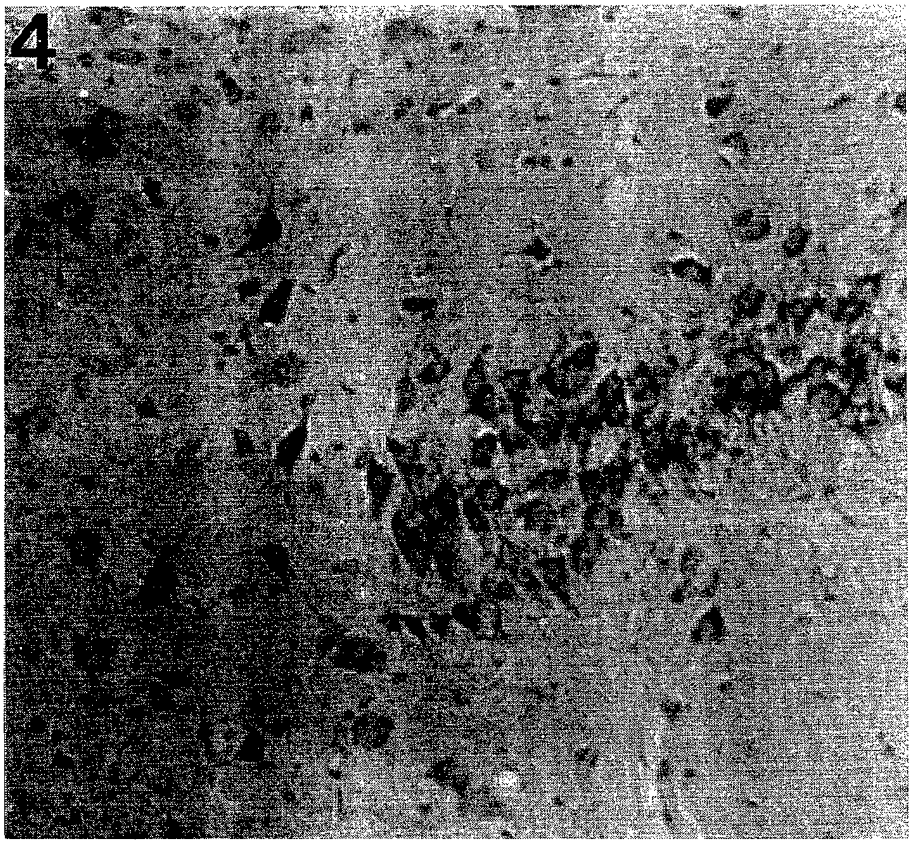
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Figure 16
High magnification of area 5, time point B.   Notice some cells near the inside right portion of this photograph beginning to show darkening. Microscope magnification, 100x.

5

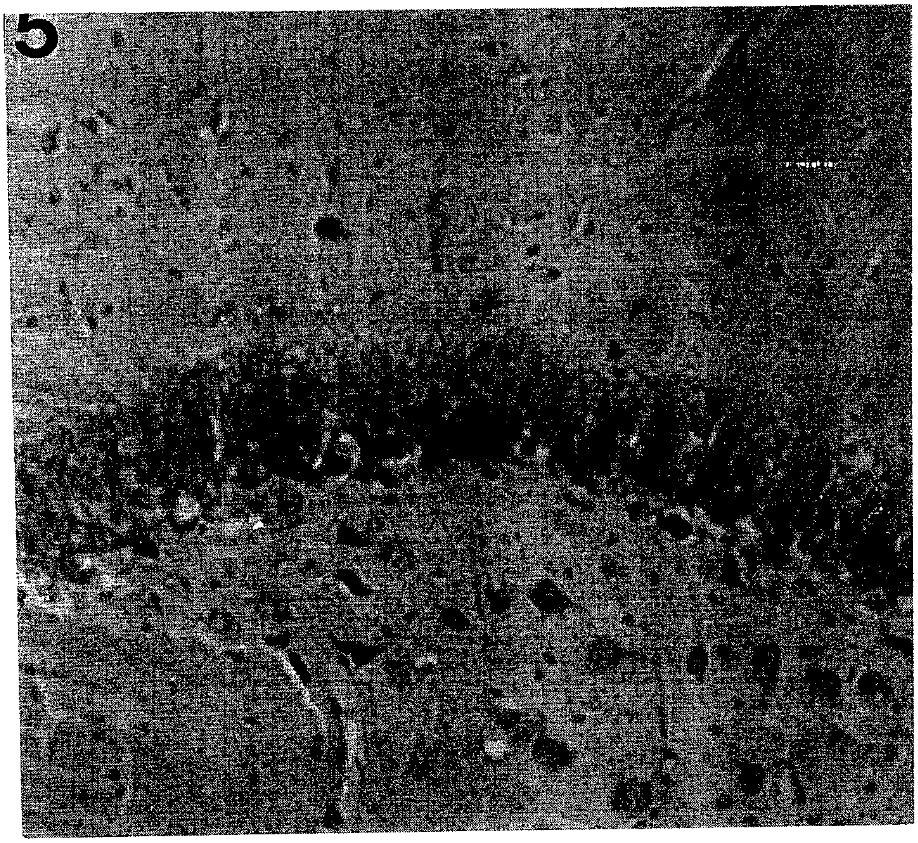

Figure 17
High magnification of area 1, time point B, stained for apoptotic nuclei.
One CA1 nucleus is TUNEL labeled indicating apoptosis.

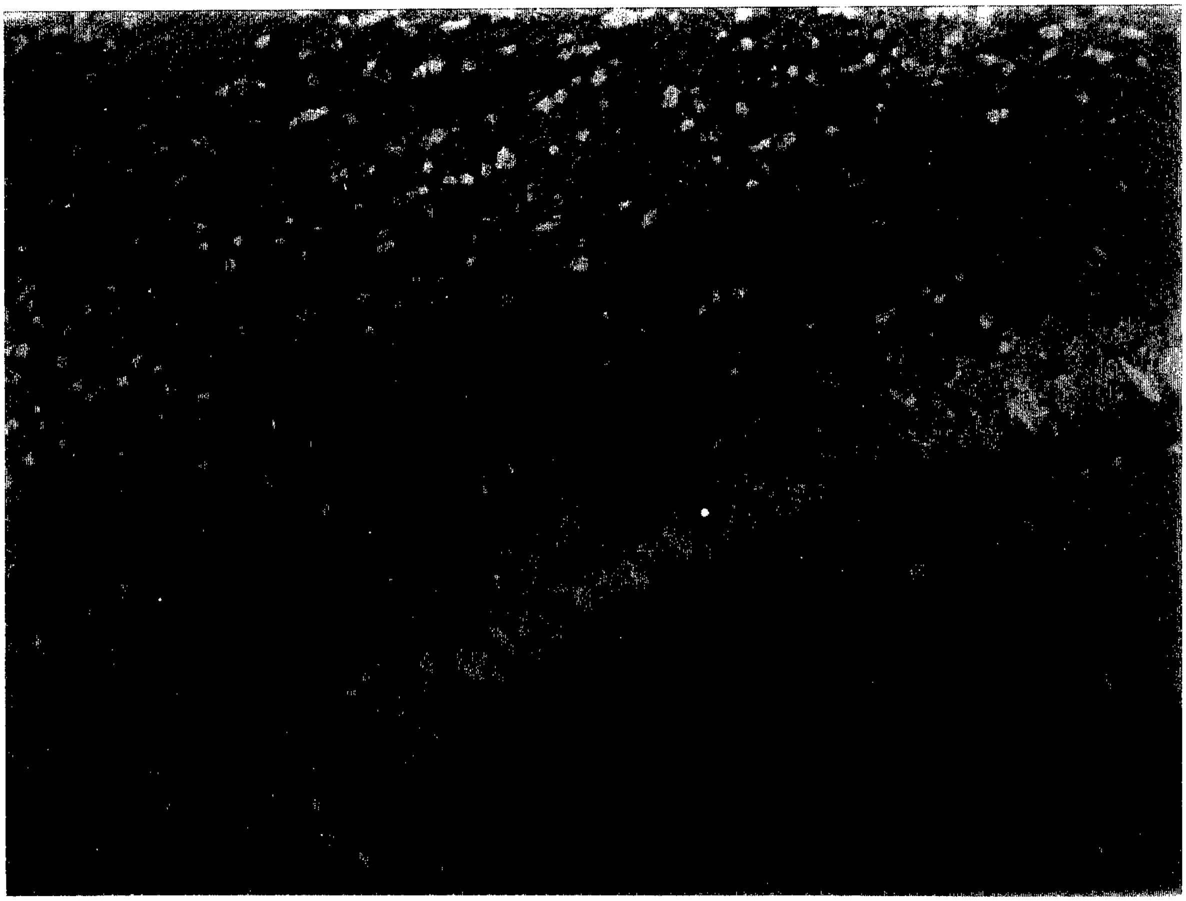

Figure 18
High magnification of area 2, time point B, stained for apoptotic nuclei.
There is no apparent apoptosis.

Figure 19
High magnification of area 3, time point B, stained for apoptotic nuclei.
Here, two apoptotic cells are visible in the CA3 region.

Figure 20
High magnification of area 4, time point B, stained for apoptotic nuclei.
One apoptotic cell is apparent here in the dentate gyrus.

Figure 21
High magnification of area 5, time point B, stained for apoptotic nuclei.
No labeled cells are visible in this region.

Figure 22
Light micrograph of control hippocampus, time point C. Many cells within the CA1, CA3, hilus, and dentate gyrus are now darkened and visibly altered. Microscope magnification, 20x.

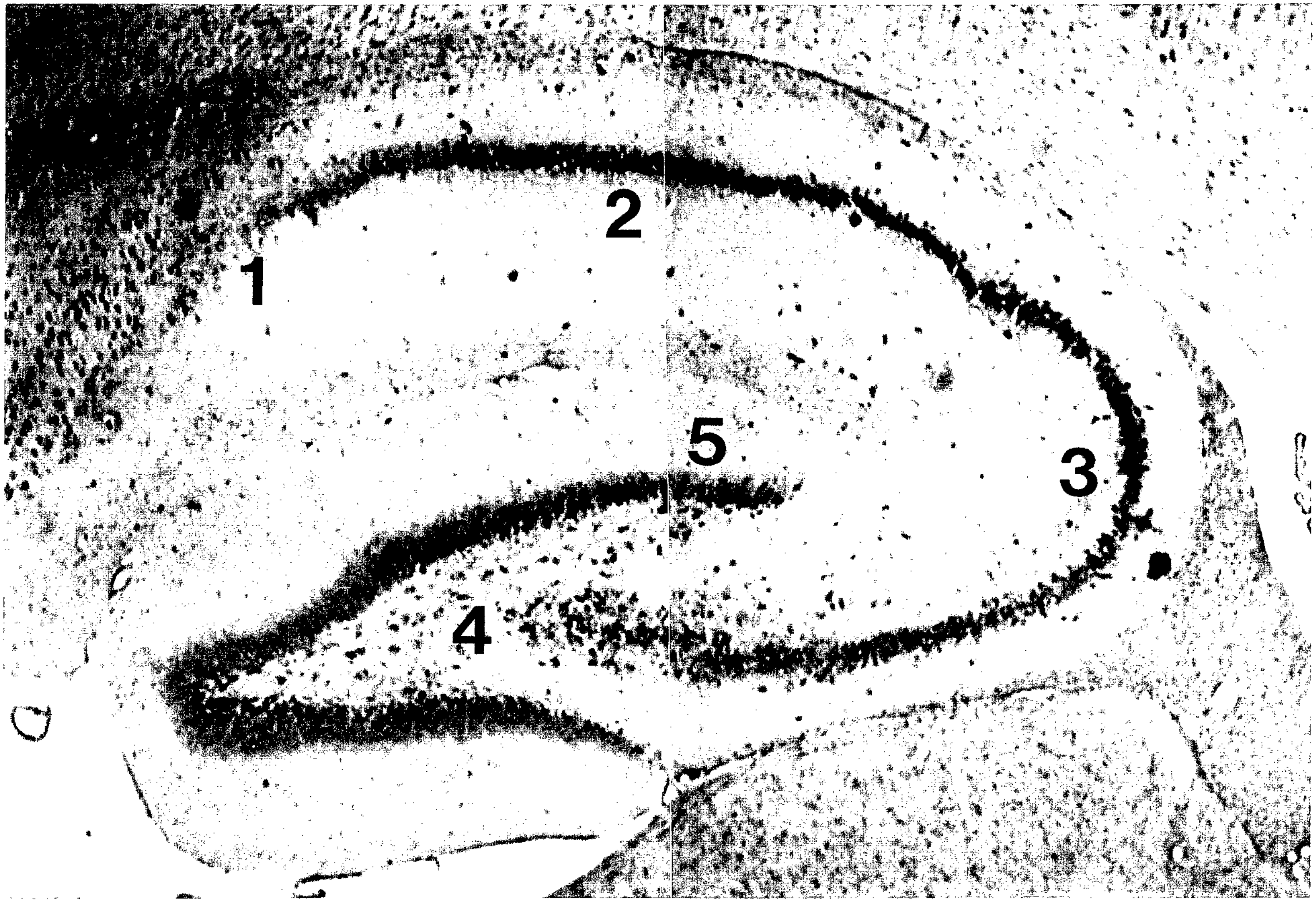
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Figure 23
High magnification of area 1, time point C. Some cell bodies appear normal (CA1 cells) while other peripheral cells have darkened. Microscope magnification, 100x.

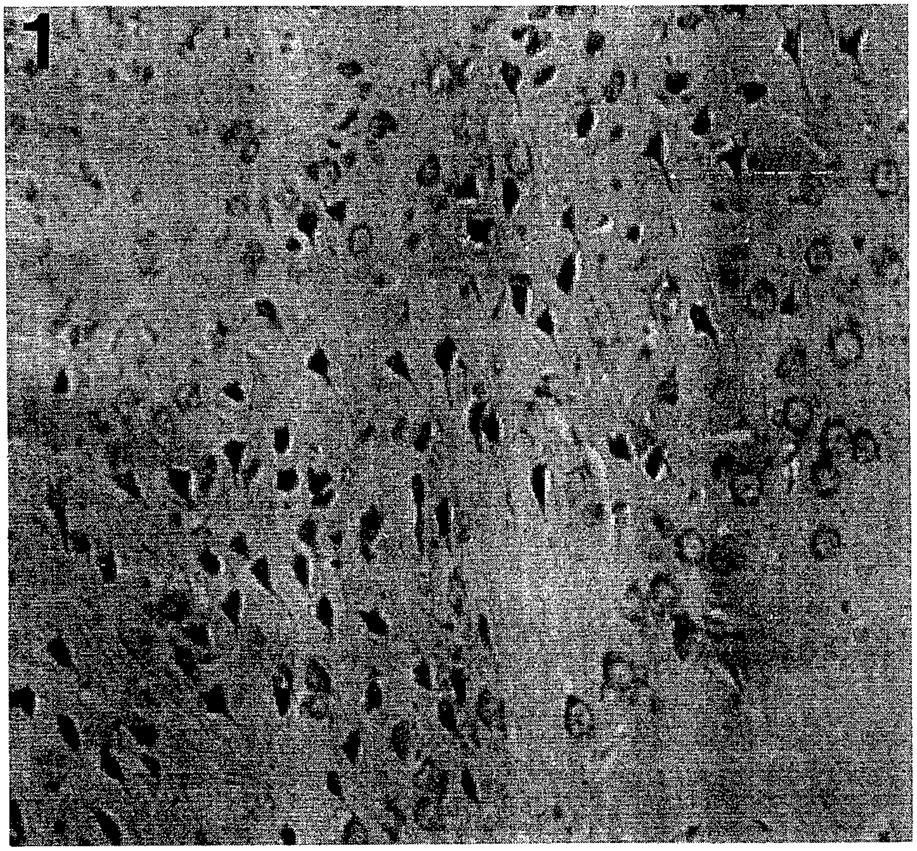
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Figure 24
High magnification of area 2, time point C. Most cell bodies within this region have now become altered. While there is no apparent cell loss, all cells have become darkened, smaller, and angular. Microscope magnification, 100x.

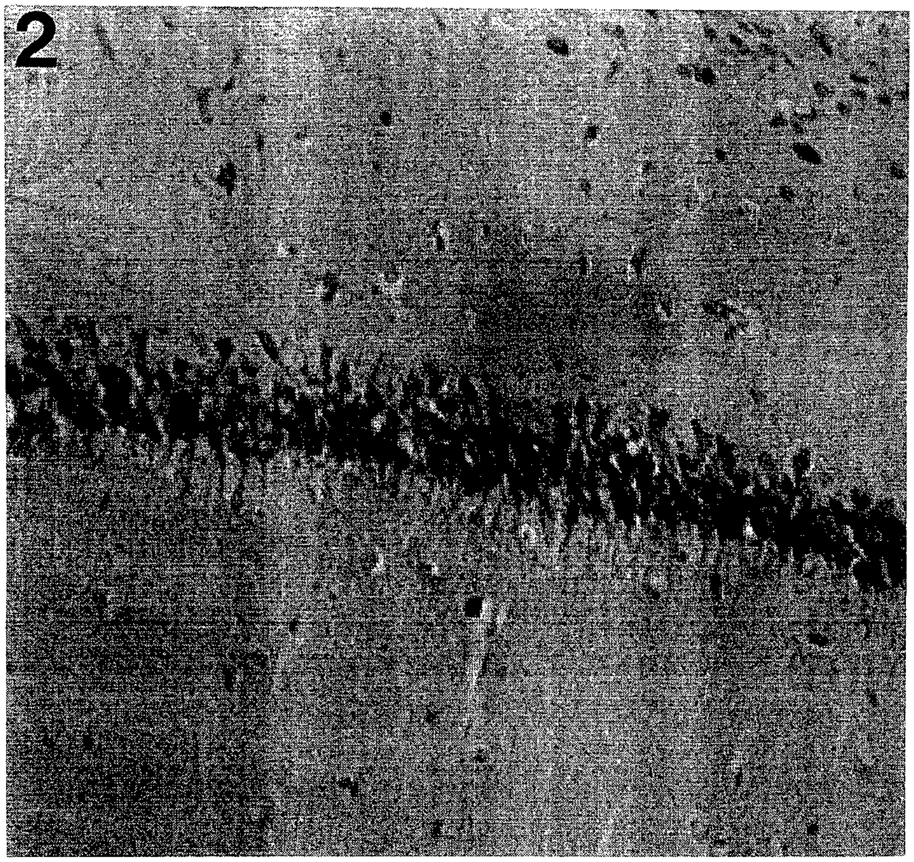
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Figure 25
High magnification of area 3, time point C. Cell bodies are now darkening and shrinking in size here. Microscope magnification, 100x.

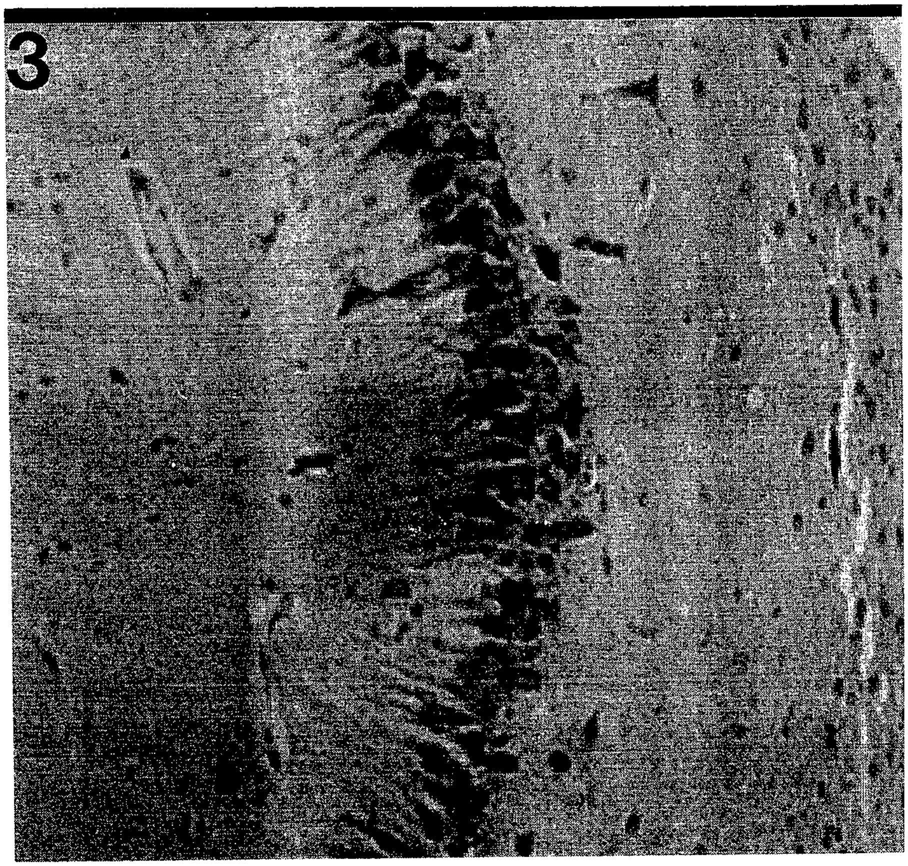
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Figure 26
High magnification of area 4, time point C. While there are some normal CA3 cell bodies here, many others have now become altered.
Microscope magnification, 100x.

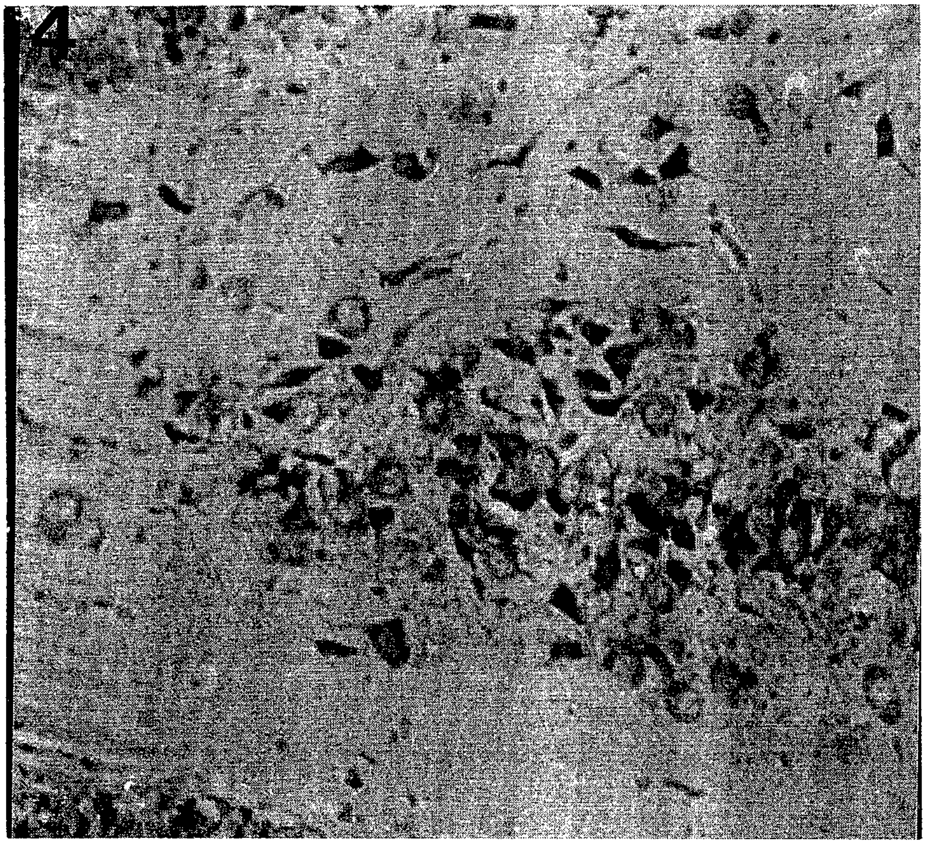
4

Figure 27
High magnification of area 5, time point C. Many cell bodies have darkened here, but mostly on the inner surface of this blade of the dentate gyrus. Some hilar cells are also affected. Microscope magnification, 100x.

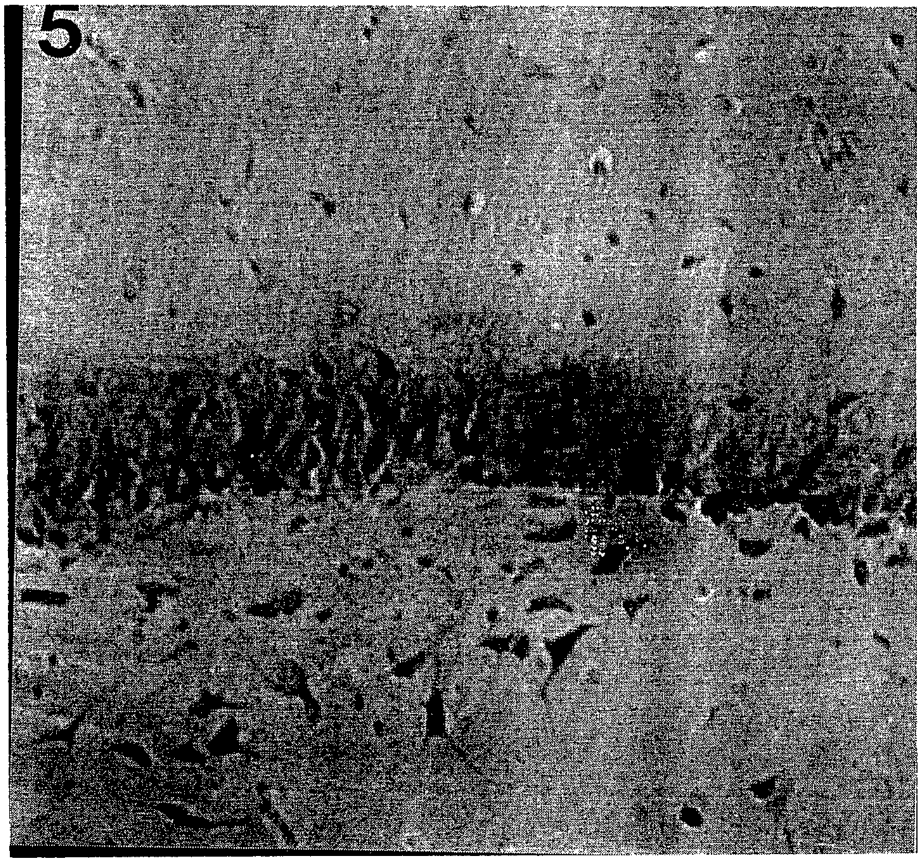
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Figure 28
High magnification of area 1, time point C, stained for apoptotic nuclei.
While many cells are affected, no cells are apoptotic.

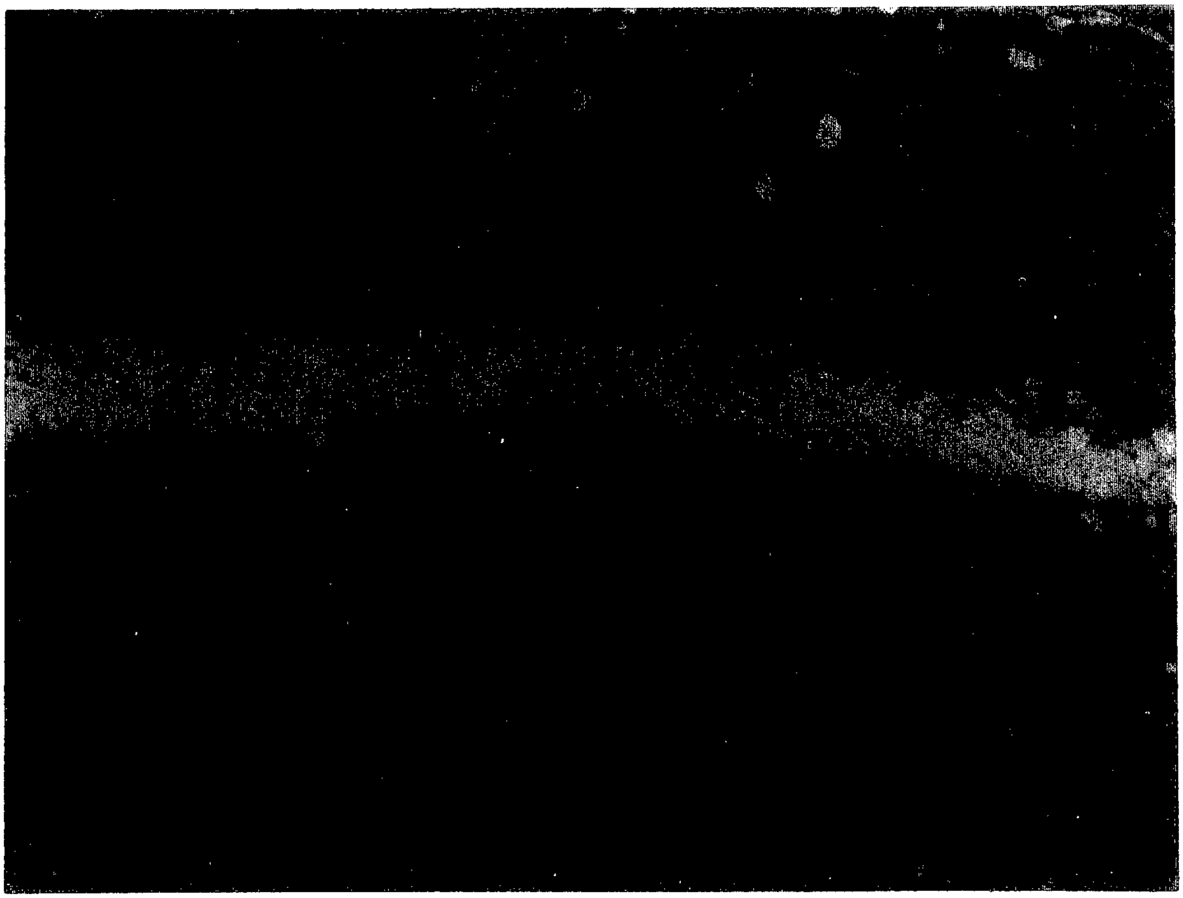

Figure 29
High magnification of area 3, time point C, stained for apoptotic nuclei. In this region, no apoptotic nuclei are seen, although 1 cell is labeled on the outer surface of the hippocampus.

Figure 30
High magnification of area 4, time point C, stained for apoptotic nuclei.
No labeled cells are present here.

Figure 31
Control hippocampal section, kianic acid triggered cell damage, stained for apoptotic nuclei.

Improved Non-Steroid Anti-Inflammatory Drugs

COX-2 Enzyme Inhibitors

Edited by

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9 An inhibitor of injury-induced COX-2 transcriptional activation elicits neuroprotection in a brain damage model

N.G. BAZAN, G. ALLAN and V.L. MARCHESELLI

The quest to understand the molecular changes in brain injury is providing new ideas that might lead to more effective therapeutic approaches. Early molecular events in brain injury, such as the hydrolysis of membrane phospholipids, can trigger cascades of events which may constitute decision pathways leading to neuronal damage or cell death, or conversely to repair and regeneration. The fundamental problems are to identify which second messengers accumulate, the signals that trigger their production and the pathways that, in turn, they affect in the damaged regions of the brain. In the design of more discriminating pharmacological approaches it is important to understand how intervention at a single point in a signalling pathway will affect the complex web of cellular communications between neurones, and between the neurone, glia and cerebral microvasculature. While the short term survival of the subject in the acute phase of stroke, head injury or epilepsy is obviously of paramount importance, an understanding of the mechanisms which lead to the alterations in synaptic circuitry involved in delayed neurobehavioural disorders such as post-traumatic epilepsy, delayed amnesia, psychosis and dementia could help in designing therapies which promote better long-term recovery.

In this chapter, we discuss the mechanisms by which lipid second messengers accumulate during brain injury, show how one of these second messengers, platelet-activating factor (PAF), is involved in the induction of the inducible cyclooxygenase (COX-2), and present ideas for pharmacological intervention in brain injury by specific modulation of this form of COX.

PHOSPHOLIPASE ACTIVATION AND ARACHIDONIC ACID RELEASE

The accumulation of biologically active lipid second messengers is an early event in brain injury[1]. Many of these changes are the result of phospholipase activation following an increased calcium influx and/or intracellular calcium mobilization in the cell[2]. While phospholipid metabolism in excitable membranes is very sensitive to brain injury[3–6], the detailed events involved in the activation of neural phospholipases under pathophysiological conditions are still not fully understood. Under normal conditions, the metabolism of arachidonoyl phos-pholipids in the CNS, regulated through phospholipase A_2 (PLA_2) and acyltransferase, favours cellular retention and maintenance of very low levels of free arachidonic acid (20:4)[7]. During brain injury there is membrane depolarization and calcium influx[8], followed by the massive release of free 20:4[9–11]. While the main release of free 20:4 occurs after a lag during which cellular conditions become favourable for phospholipase activation, in cerebral ischaemia 20:4 accumulates gradually as brain ATP levels drop, but before membrane depolarization. This suggests that in addition to phospholipase activation, accumulation of free 20:4 also involves a down-regulation of energy-dependent reacylation into membrane phospholipids[8].

Two enzymatic pathways have been suggested as contributors to 20:4 release during the rapid early phase of free fatty acid (FFA) accumulation: PLA_2-mediated deacylation of phospholipids and the sequential degradation of inositol lipids by phospholipase C-diacylglycerol (DAG) and monoacylglycerol lipases[7,12]. An additional mechanism may be the activation of a phospholipase D (PLD) that hydrolyses phosphatidylcholine to release phosphatidic acid; this, in turn, is acted upon by phosphatidate phosphohydrolase[12,13] to yield DAG.

Elevated PLA_2 activity in brain has been reported during ischaemia and head injury[14–16]. However, the generation of free 20:4 through the PLC–DAG lipase pathway during ischaemia and seizures is supported by the observation that accumulation of free 20:4 coincides with the release of 20:4–DAG through the phosphodiesteratic degradation of polyphosphoinositides[4,17–19]. However, dissociation between 20:4 accumulation and DAG release was observed in the anoxic immature murine brain, where free 20:4 accumulates after a prolonged lag period with no accompanying release of 20:4–DAG[20]. It is likely that more than one phospholipase is responsible for 20:4 release during brain trauma. Questions regarding the timing and sequence of phospholipase activation, and the phospholipid pools from which individual membrane-derived lipid second messengers are derived, remain to be answered. Analysis of the kinetics of inositol phospholipid degradation and accumulation of free 20:4 and 20:4–DAG in rat brain following a single electroconvulsive shock (ECS) suggests that activation of the individual phospholipases is time-dependent and that different phospholipid pools are involved[19,21]. During the tonic phase of the seizure, the fall in 20:4 content in PPI is accounted for by the increase of 20:4 in free fatty acids and DAG. Later, during the clonic phase, degradation of other 20:4-containing phospholipids contributes to the increased 20:4 content in the free fatty acid pool. The tonic-phase accumulation of PPI-derived second messengers appears to involve activation of both PLC and PLA_2, as treatment of rats with *Ginkgo biloba* extract prior to ECS blocks the release of 20:4–DAG in the hippocampus without affecting free 20:4 accumulation[22]. The sequential contribution of the PLC/DAG lipase pathway, followed by PLA_2, to the accumulation of free 20:4 in the mouse brain after post-decapitation ischaemia has also been reported[23,24]. During global ischaemia of the rat brain, the inositol phospholipid pool is the main contributor to free fatty acid release via the PLC/DAG-lipase pathway. After longer times of ischaemia (2–4 min), the calcium-dependent PLA_2 pathway prevails, releasing fatty acids from phosphatidylcholine (PC) and phosphatidylethanolamine (PE)[25].

Several mechanisms may contribute to phospholipase activation during brain trauma. The primary mechanism is thought to be an elevated cytosolic concentration of Ca^{2+} triggered either by IP_3-mediated calcium mobilization from intracellular stores or by influx of extracellular calcium through plasma membrane channels[2,13,26]. Perhaps the most significant means by which intracellular calcium concentrations are raised during brain trauma is through activation of the NMDA receptor calcium channel. The massive release of glutamate under these conditions leads to an overload of intracellular Ca^{2+} and the stimulation of Ca^{2+}-dependent lipases, protease and nucleases, resulting in glutamate neurotoxicity. PLA_2-mediated release of 20:4 is triggered by NMDA receptor activation in primary cultures of striatal neurones and cerebellar granule cells[27,28], and in hippocampal slices[29], and is blocked by PLA_2 inhibitors[30]. The main PLA_2 responsible for 20:4 release in the CNS could be a high molecular weight cytosolic PLA_2 that undergoes membrane translocation upon an increase in intracellular Ca^{2+} [16,31], and which has a substrate specificity for 20:4-containing phospholipids[32,33].

Agonist interaction with receptor–G protein complexes provides another mechanism of PLA_2 stimulation. Dissociation of the heterotrimeric G proteins in α and $\beta\gamma$ subunits mediates the activation of phospholipases: the α subunit stimulates PLC while the $\beta\gamma$ subunits have been shown to activate PLA_2 in retinal rod outer segments[34,35]. The high homology between PLA_2 activating protein (PLAP) and β-transducin[36] implicates the G protein signalling pathway in the direct activation of PLA_2.

The release of 20:4 may also be modulated by lipocortins (annexins), a family of calcium and phospholipid binding proteins that inhibit PLA_2. While, as discussed later in this chapter, glucocorticoids are known to inhibit induction of the inducible prostaglandin synthase, their anti-inflammatory properties have also been linked to their ability to induce lipocortin synthesis[37]. Steroid-inducible lipocortin-1 is present in neuronal and glial cells, especially in the hippocampus[38], where it is thought to be an endogenous neuroprotective agent[39]. When administered intraventricularly to rats, the protein significantly reduces infarct size and oedema induced by cerebral ischaemia[40] and attenuates excitotoxic damage mediated by NMDA receptors[41].

ROLES OF ARACHIDONIC ACID IN NEURONAL PHYSIOLOGY AND PATHOPHYSIOLOGY

Release of 20:4 plays a central role in the development of brain injury. Eicosanoids synthesized from free 20:4 are potent mediators of inflammation, increasing vascular permeability and promoting the development of brain oedema[42]. It should not be forgotten, however, that the free fatty acid itself is a significant modulator of neuronal activity, acting on a variety of functional membrane proteins, including receptors, ion channels and enzymes[43–45].

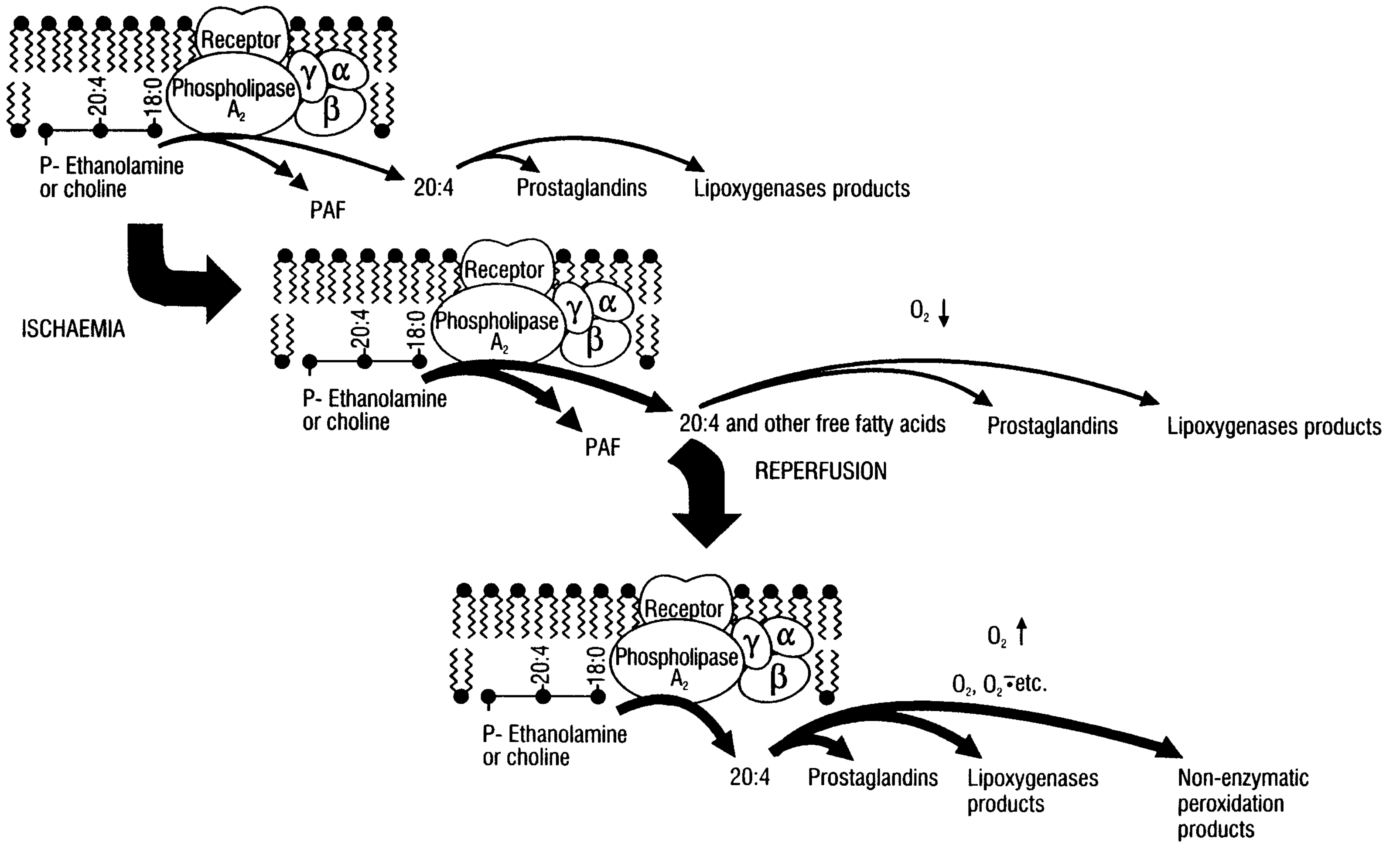
The NMDA receptor-stimulated release of 20:4 results in perturbations of ionic homeostasis and alterations in neuronal excitability. Arachidonic acid-mediated activation of potassium channels leads to neuronal hyperpolarization and synaptic depression[43]. However, neuronal excitability is promoted by inhibition of glutamate uptake[46,47], stimulation of glutamate release[48,49], activation of A_1-adenosine receptors[50], and increasing the open-channel probability of the NMDA receptor[51]. While it is not always possible to distinguish which of these effects are due to the direct action of the fatty acid, analysis of the NMDA receptor amino acid sequence shows a domain homologous to fatty acid-binding proteins[52] and thus supports a direct interaction of free 20:4 with the NMDA receptor. Arachidonic acid may also stimulate glutamatergic neurotransmission, probably acting as a retrograde messenger at the presynaptic level, stimulating further release of glutamate. These pre- and post-synaptic effects may contribute to long lasting changes in glutamate synaptic efficacy or long term potentiation (LTP), a form of synaptic plasticity essential to the process of memory and learning in the brain[53]. This is discussed in more detail below.

PROSTAGLANDIN SYNTHESIS IN BRAIN DAMAGE

In the early stages of brain damage, the release of 20:4 is the rate-limiting step in the synthesis of prostaglandins, potent modulators of neuronal function[42,54]. The molecular mechanism of ischaemia/reperfusion-induced neuronal injury involves the release of 20:4 during the ischaemic phase and its conversion to prostaglandins[55,56] and leukotrienes[57,58] during reperfusion (Figure 1). These metabolites can elicit a variety of effects, not only in neural cells but also in the microvasculature: prostaglandins affect cerebral blood flow[59] and leukotrienes increase blood–brain barrier permeability[60,61].

The entry point of free 20:4 into the prostaglandin synthesis pathway is the cyclooxygenation of 20:4 to PGG_2 followed by its hydroperoxidation to PGH_2, catalysed by prostaglandin synthase (COX, cyclooxygenase endoperoxide synthase, PGH synthase, EC 1.14.99.1). For a long time it was thought that there was only a single, constitutive COX, and that the increased synthesis of prostaglandins in pathophysiological situations simply reflected the increased availability of substrate. There is now known to be two forms of COX, a typically constitutive enzyme (COX-1) insensitive to glucocorticoids, and an inducible enzyme (COX-2), expressed in response to mitogen stimulation and sensitive to glucocorticoid inhibition[62,63]. COX-2 was originally cloned as one of a series of primary response genes induced in Swiss 3T3 cells by tetradecanoyl phorbol acetate (TPA) known as TPA-induced sequences (TIS genes)[64], and as a mitogen-

Figure 1 ***Free arachidonic acid release, eicosanoid formation and lipid peroxidation during brain injury, as illustrated by ischaemia–reperfusion. Under physiological conditions there is a low level of receptor-mediated PAF and eicosanoid synthesis. During the ischaemic phase, there is an overactivation of phospholipase A2 and an accumulation of free arachidonic acid. The lowering of oxygen tension and metabolic energy impairs synthesis of eicosanoids and the re-esterification of arachidonic acid into membrane phospholipids. Reperfusion leads to a rise in oxygen tension and a massive burst of oxidative metabolism and synthesis of eicosanoids. A by-product of oxidative metabolism is superoxide and other oxygen radicals. Their accumulation under these conditions can exceed the capacity of the cellular free radical scavenging and antioxidant mechanisms, leading to lipid peroxidation and other manifestations of oxidative stress***


Receptor
Phospholipase A_2
γ
α
β
20:4
18:0
P- Ethanolamine or choline
PAF
20:4
Prostaglandins
Lipoxygenases products
ISCHAEMIA
Receptor
Phospholipase A_2
γ
α
β
20:4
18:0
P- Ethanolamine or choline
PAF
O_2 ↓
20:4 and other free fatty acids
Prostaglandins
Lipoxygenases products
REPERFUSION
Receptor
Phospholipase A_2
γ
α
β
20:4
18:0
P- Ethanolamine or choline
O_2 ↑
O_2, $O_2^{\bullet -}$ etc.
20:4
Prostaglandins
Lipoxygenases products
Non-enzymatic peroxidation products

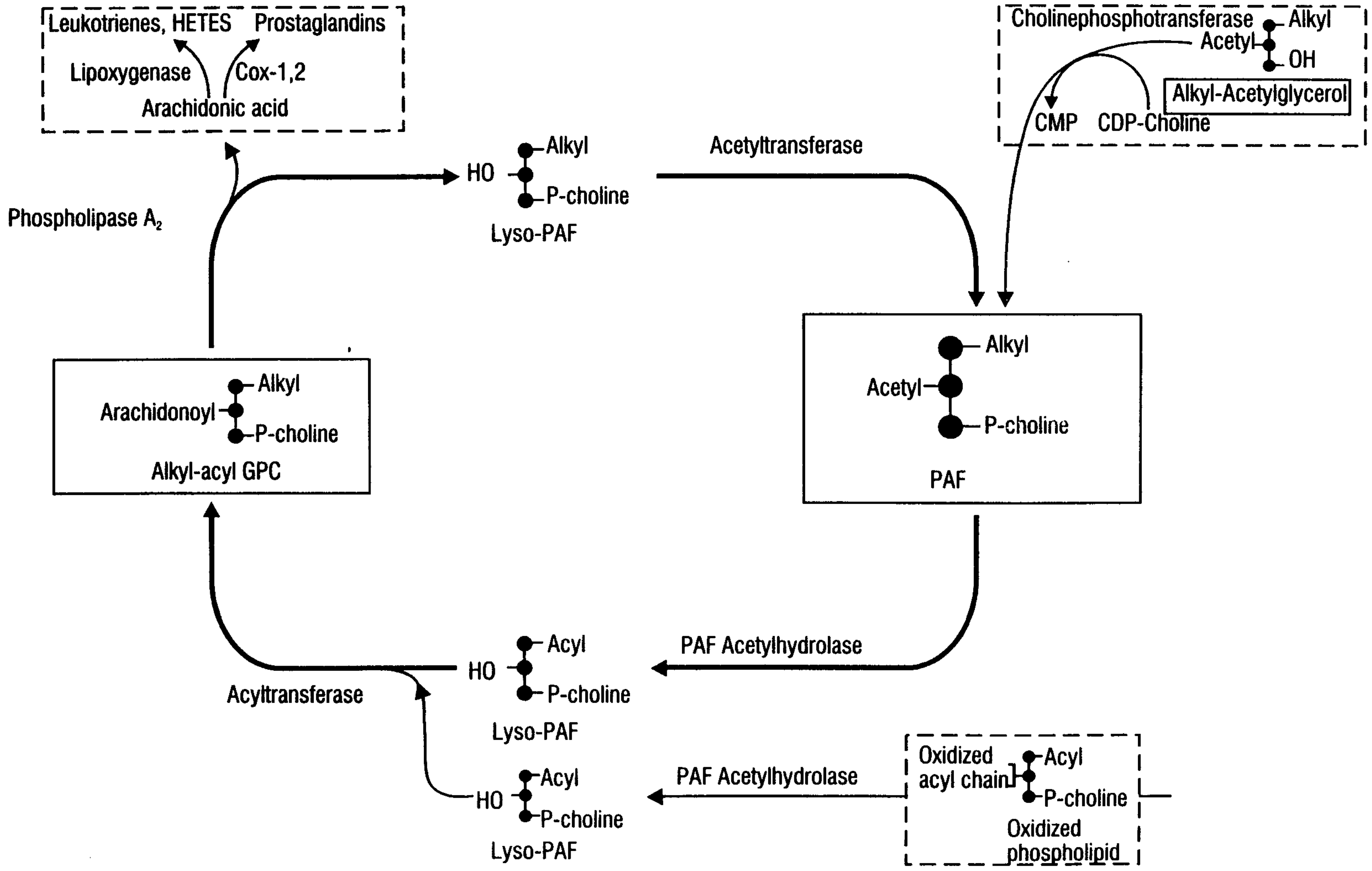
inducible gene in chick embryo fibroblasts[65]. In the brain, COX-2 is expressed mainly in cortical and limbic neurones but not in glia or vascular endothelium[66,67]. NMDA-dependent synaptic activity implicated in memory and learning processes as well as in excitotoxic neuronal damage has been shown to regulate the expression of the COX-2 gene[66]. Antibodies against COX-2 stain primarily dendrites and cell bodies[67], implying post-synaptic localization. This suggests that prostaglandins may play a role in NMDA-mediated neuronal plasticity and, under pathological conditions (i.e. ischaemia, stroke), in neural cell injury and death.

PLATELET-ACTIVATING FACTOR AND BRAIN

PAF (1-*O*-alkyl-2-acetyl-glycero-3-phosphocholine) is an acetylated alkyl ether phospholipid originally described as a mediator released by leukocytes which induces the aggregation and activation of rabbit platelets[68,69]. It is now known to have much broader functions as a mediator of inflammatory and immune responses, and as a second messenger involved in intracellular communication[70–72]. PAF is found in the central nervous system, including in brain and retina[73–75]. It is rapidly produced in brain in response to ischaemia and seizures[73,76] and in neuronal cell cultures in response to neurotransmitters[77]. PAF can accumulate in the brain in the absence of inflammatory cells, which implies that some or all of the endogenous cell types in the CNS are capable of synthesizing PAF.

A small, but metabolically active pool of 1-*O*-alkyl-2-arachidonoyl-glycero-3-phosphocholine (alkylacyl-GPC), is an important phospholipid component of excitable membranes. Its hydrolysis, catalysed by PLA_2, yields both 20:4, the precursor of eicosanoids, and lyso-PAF, a direct precursor of PAF. Alkylacyl-GPC is a substrate for a cytosolic PLA_2 that is present in the brain and is activated and translocated to the membrane following an increase in intracellular calcium levels[16,31]. As a result, lyso-PAF becomes available for transacetylation by acetyl CoA:lyso-PAF acetyltransferase to yield biologically active PAF. This route of PAF synthesis is known as the remodelling pathway (Figure 2). There are at least two alternative pathways by which PAF can be generated: the de novo synthesis route and a CoA-independent transacylase. The de novo synthesis pathway involves the synthesis of 1-*O*-alkyl-2-acetylglycerol, which is then converted to PAF by the cholinephosphotransferase-catalysed transfer of a choline moiety from CDP-choline. The other pathway consists of an alternative route for the generation of lyso-PAF via the CoA-independent transacylation of 20:4 acid from alkylarachidonoyl-GPC to an acceptor lysophospholipid. The contribution of these pathways to PAF synthesis during neuronal stimulation has yet to be fully evaluated, although the full complement of enzymes required for all pathways are present in brain[78,79]. Acetylcholine and dopamine stimulate PAF synthesis in developing retina via the de novo pathway[75], and the brain has high

Figure 2 *The PAF cycle. The membrane phospholipid precursor of PAF, alkylacyl-GPC, is cycled through lyso-PAF, PAF, and after inactivation by PAF acetylhydrolase is reincorporated into the membrane phospholipid pool. The de novo pathway (top right) is independent of this cycle, but there can be input into the lyso-PAF pool from alkylacyl-GPC which has undergone lipid peroxidation and fragmentation of the polyunsaturated acyl chain. The resulting PAF-like molecule is then a substrate for PAF acetylhydrolase. Not shown here is the transacylase pathway, in which transacylation of the arachidonoyl chain of alkylacyl-GPC to an acceptor lysophospholipid leads to formation of lyso-PAF*


Leukotrienes, HETES
Prostaglandins
Lipoxygenase
Cox-1,2
Arachidonic acid
Cholinephosphotransferase
Alkyl
Acetyl
OH
Alkyl-Acetylglycerol
CMP
CDP-Choline
Phospholipase A_2
HO
Alkyl
P-choline
Lyso-PAF
Acetyltransferase
Arachidonoyl
Alkyl
P-choline
Alkyl-acyl GPC
Acetyl
Alkyl
P-choline
PAF
HO
Acyl
P-choline
Lyso-PAF
PAF Acetylhydrolase
Acyltransferase
HO
Acyl
P-choline
Lyso-PAF
PAF Acetylhydrolase
Oxidized acyl chain
Acyl
P-choline
Oxidized phospholipid

levels of 1-*O*-alkyl-glycero-3-phosphate:acetyl CoA acetyltransferase, which catalyses the synthesis of 1-*O*-alkyl-2-acetylglycerol (the direct precursor of PAF in the de novo pathway)[80]. However, the rise in intracellular calcium which accompanies activation of many receptors, including the NMDA class of glutamate receptors, and the disruption of ion homeostasis in cerebral trauma[81] inhibits the phosphocholine transferase required in the de novo pathway[82].

High-affinity PAF binding sites are found in synaptosomal and microsomal membrane subcellular fractions of rat cortical homogenates[83]. The two sites differ in the kinetics of [^{3}H]PAF binding: the microsomal membranes in fact display cold-ligand displacement kinetics characteristic of two binding sites. They also show differential sensitivity to PAF receptor antagonists. The most experimentally useful of these drugs are BN 52021, a terpenoid extracted from the leaf of the *Ginkgo biloba* tree which binds preferentially to the synaptosomal site[83], and the hetrazepine BN 50730, which shows complete specificity for the microsomal sites. The synaptosomal site appears to correspond to the cloned PAF receptor[84–86], which is a member, along with receptors for other lipid mediators such as prostacyclin[87], of the seven membrane-spanning domain, G protein-coupled receptor superfamily. Of the two transcripts produced from this gene (1 and 2), only type 1 is expressed in brain[88]. Multiple signal transduction pathways can be stimulated by activation of the PAF receptor (Figure 2). PAF treatment of hippocampal neurones in culture elicits intracellular calcium fluxes, the dynamics of which are characteristic of release from intracellular stores rather than gating of extracellular calcium[89]. This correlates with evidence that the primary mode of signal transduction for the cell surface PAF receptor is via a G protein-activated phospholipase C[90,91]. PAF is also reported to down-regulate adenyl cyclase via a pertussis toxin-sensitive mechanism[92] in some cells, to induce extracellular calcium influx[93] and to activate the microtubule-associated protein kinase (MAP kinase) and other protein kinase cascades[92,94]. The identity of the BN 50730-sensitive receptors(s) remains unclear, as does their cellular origin (neurones, glia or microvascular endothelium). It is apparent, however, that the two classes of receptor differ in the signal transduction mechanisms to which they are coupled.

There is evidence to suggest that PAF is a mediator of signal transcription coupling that may, in turn, be involved in neuronal plasticity, remodelling of synaptic circuitry and epileptogenesis. PAF rapidly and transiently augments levels of the c-*fos* and c-*jun* transcription factor mRNAs in a neuronal cell line[95]. Phorbol esters and PAF synergistically stimulate c-*fos* expression, suggesting that the transcriptional effects of PAF are not mediated by protein kinase C. The effect of PAF is at the transcriptional level, as opposed to increasing the stability of the mRNA 5′ deletion; mutagenesis studies of the c-*fos* promoter show that the calcium-response element is necessary for the PAF-induced response. There are other examples of PAF-induced gene expression in neuronal and non-neuronal cells, including the heparin-binding epidermal growth factor in monocytes[96], c-

fos and *egr-2* in lymphoblastoid cell lines[97], the transcription factor NF-κ B and immunoglobulins[98] in human B cell lines, c-*fos* and zif/268 in rat astroglia[99], and c-*fos* and TIS 1 in A-431 epidermoid carcinoma cells[100]. There are also reports that PAF is able to autoregulate gene expression of its own cell surface receptor[101,102].

PAF ENHANCES COX-2 PROMOTER ACTIVITY IN VITRO

COX-1 and COX-2 are encoded for by distinct, but evolutionarily related, genes. The human and mouse COX-1 genes contain 11 exons and 10 introns, and span about 22 kb of DNA[103,104]. The human, mouse and chicken COX-2 genes contain 10 exons and 9 introns[105–107] and span only 8–9 kb of DNA. Exons 1–9 of COX-2 are homologous to exons 2–10 of COX-1, and thus both enzymes include haem ligand sites, glycosylation sites, aspirin acetylation sequence, and an epidermal growth factor homology region. The COX-2 gene, however, lacks the exon present in the COX-1 gene that encodes a hydrophobic N-terminal region. The 3′ exon of COX-2 encodes an additional 18 amino acid region in the C-terminal region of COX-2, and a significantly longer 3′-untranslated region in the mRNA which includes several AUUUA sequences characteristic of short-lived messages.

The COX-2 promoters from human[108], rat[109], mouse[105] and chicken[106] have been cloned and analysed. There are some species differences in the organization of promoter elements, but, in the mouse promoter, a number of responsive elements for different inducers of COX-2 expression have been tentatively identified, including v-*src*[106], gonadotrophic hormone[109] and, most significantly in the context of this chapter, PAF[110].

PAF can induce mouse COX-2 promoter activity when constructs are transfected into neuronal (NG108-15 neuroblastoma×glioma hybrid) and non-neuronal (NIH-3T3) cells. When constructs containing the proximal 371 bases (−371 to −1) of the mouse COX-2 promoter are transfected into cells using the calcium phosphate co-precipitation procedure, in the presence of retinoic acid (100 nM), there is dose-dependent (1–50 nM) PAF-induced expression of the luciferase reporter gene. When the vector is transfected into the cells using cationic liposomes (lipofection), reporter gene expression can be induced by PAF alone. There is also activity from constructs containing the proximal 963 bases of the mouse COX-2 promoter, although this is less, suggesting the presence of inhibitory sequences upstream from −371. The effect is rapid, with some activity after as little as 15 or 45 min of incubation with the ligands. This is not sufficient time for de novo protein synthesis, indicating that COX-2 promoter activation in this system involves the modulation of pre-existing transcription factors. Preincubation of the cells with the intracellular PAF antagonist BN 50730 inhibits the PAF/RA induction of luciferase reporter gene expression, which supports this effect as being a specific receptor-mediated phenomenon, and indicates that it is mediated through the intracellular PAF receptor. The use of constructs with 5′ deletions of this promoter in similar transfection experiments narrows down the region of the promoter containing the 'PAF-responsive' element to between −371 and −300; deletion of this region lowers PAF induction from 31 times to 4.1 times control levels. More detailed mapping of this region and the identification of the transcription factors responsible for the PAF regulation of COX-2 are the subjects of ongoing research.

EXPERIMENTAL SEIZURES PROMOTE SUSTAINED TRANSCRIPTIONAL ACTIVATION OF COX-2 IN HIPPOCAMPUS

The PAF antagonist BN 50730 inhibits COX-2 gene expression in the brains of rats subjected to two types of experimental seizures, a single ECS which elicits a single, generalized tonic-clonic seizure or seizures induced by kainic acid (KA), a conformationally rigid analogue of glutamic acid which is an agonist of the AMPA-kainate subclass of glutamate receptors. Injection of KA, either parenterally or intracerebrally, induces limbic seizures similar to the complex partial seizures seen in human temporal lobe epilepsy. In addition, there is a loss of pyramidal cells and damage to the CA3 region of the hippocampus which results in sprouting of mossy fibres from the dentate gyrus. The brain damage produced is similar to that which occurs in the human epileptogenic condition of Ammon's horn sclerosis[111].

A single pretreatment of the animals by intracerebroventricular injection with BN 50730 is sufficient to block most of the COX-2 mRNA accumulation in both seizures models (Marcheselli VL and Bazan NG, submitted for publication), and is a more effective inhibitor of COX-2 induction than the glucocorticoid dexamethasone. This implies that PAF accumulation and activation of the intracellular PAF receptor leads to the majority of the induced prostaglandin synthesis under these conditions. Interestingly, the level of induction of COX-2 and other immediate early genes is much greater and more prolonged in the KA than in the ECS model. This may be reflected in the more profound long-term effects of the KA treatment.

The consequences of inhibiting COX-2 and other PAF-mediated effects on the progression of KA-induced brain lesions is the subject of ongoing research. There is, however, another model of brain injury, cryogenically induced vasogenic cerebral ischaemia, in which inhibition of COX-2 expression correlates with an abrogation of the oedema.

VASOGENIC CEREBRAL OEDEMA INDUCES COX-2

Application of a liquid nitrogen-cooled metal probe to the exposed skull of an experimental animal produces a localized lesion in the parietal cortex, and oedema in the ipsilateral, and to a lesser degree in the contralateral, cerebral hemisphere[112]. The oedema results from the increased permeability of the cerebral microvasculature and the accumulation of fluid in brain tissue. Within the closed, rigid structure of the skull, the resultant swelling leads to increased hydrostatic pressure on the brain, and provides an animal model of the life-threatening consequences of brain oedema seen in humans with closed head injury.

In the cryogenic injury model, the lesion results in the transient accumulation of COX-2 mRNA and protein in the ipsilateral cerebral hemisphere (Marcheselli VL and Bazan NG, submitted for publication). Up-regulation of COX-2 mRNA and protein expression following the insult is strongly inhibited by pretreatment of the animals with a single intracerebroventricular injection of BN 50730, and to a less, but still significant extent, by treatment with dexamethasone. Drug treatment also drastically reduces the brain oedema, and the relative efficacy of the PAF antagonist and the glucocorticoid correlates with their ability to inhibit COX-2 induction. These findings provide a correlation between PAF receptor activation in the brain, induction of COX-2 expression, and increased permeability of the cerebral microvasculature. This suggests that either PAF antagonists or COX-2 inhibitors might be useful drugs in protecting against the formation of cerebral oedema within 24h or so following head injury. It may, however, be the case that accumulation of PAF and/or prostaglandins has a role in the long term survival of neurones and retention of normal synaptic pathways following this type of brain trauma. In this case, the use of either PAF antagonists or COX-2 inhibitors might alleviate the oedema, but may also potentiate long term neuronal damage. Alternatively, while the PAF/COX-2 pathway might be involved in oedema, other pathways may contribute to neuronal damage and pathophysiological synaptic plasticity. If this were so, then adjuvant therapy with other drugs might be needed to combat both the cerebral ischaemia and the neuronal damage in individuals with head injury.

PAF INDUCES COX-2 EXPRESSION IN CORNEAL EPITHELIUM

PAF has been shown to induce COX-2 gene expression in a non-neuronal tissue, the corneal epithelium. The cornea provides a transparent aperture through which light can reach the retina, and provides much of the eye's refractive power. As the integrity of the cornea is obviously essentially for normal vision, it is acutely sensitive to traumatic and irritative insults. Although avascular, the cornea is the most richly innervated tissue in the whole body. The outer few layers of cells comprise the corneal epithelium. This responds very rapidly to damage, initially by cells migrating and spreading to cover the wound, followed by cell division to re-form the stratified epithelium.

PAF accumulates in the rabbit cornea in response to alkali burn, the amount accumulated being dependent on the severity of injury[112]. In the rabbit isolated cornea, treatment with a calcium ionophore elicits PAF synthesis, and the primary PC species from which arachidonic acid is released upon wound healing is the PAF precursor alkylacyl-GPC[113]. This implicates the remodelling pathway in wound-induced PAF release.

Endogenous PAF administered to rabbit corneas in organ cultures induces the BN 50730-sensitive expression of the immediate early genes c-*fos*, c-*jun*, urokinase plasminogen activator (uPA), and COX-2[114–116], and of the secondary response genes matrix metalloproteinases (MMP) 1 (type 1 collagenase) and 9 (gelatinase)[114,117]. Levels of COX-2 protein are elevated 4h after PAF exposure and remain elevated after 16h. Expression of matrix MMP is an important step in wound healing, as modifications of the extracellular matrix are involved in cellular migration. Although the wound can close within 24h, levels of MMP activity remain elevated for many days[118]. In addition, excessive activity of proteinases is thought to be responsible for the destruction of the extracellular matrix involved in corneal ulceration.

Prostaglandins are important regulators of MMP expression in a range of cell and tissue types, up-regulating MMP expression in osteosarcoma cells[119] and monocytes[120], but down-regulating expression in rabbit chondrocytes[121] and synoviocytes[122]. PAF-induced COX-2 expression and prostaglandin synthesis could therefore play a role in the regulation of MMP expression during corneal wound healing, and could conceivably be involved in other pathophysiological manifestations of collagen metabolism such as certain forms of arthritis.

PAF IS A MODULATOR OF NEURONAL PLASTICITY

In experimental models, PAF enhances glutamate release in hippocampal neurons in vitro[123], possibly acts as a presynaptic modulator (retrograde messenger) in hippocampal LTP[124] and is involved in the formation of certain types of memory in the limbic system[125,126] and in the caudate nucleus (Packard MG, Teather L, Bazan NG, submitted for publication). Each of these effects is inhibited by the synaptic PAF receptor antagonist BN 52021. LTP is a phenomenon in which a sustained high-frequency presynaptic stimulation of excitatory amino acid neuronal pathways results in a prolonged enhanced postsynaptic response. LTP, as a use-dependent strengthening of synaptic connections, is considered to be a simple model of the neurophysiological processes underlying memory[53]. As there is some evidence that LTP may underlie hippocampal-based learning[127], the role of PAF in LTP and learning models could be accounted for by the activity of PAF on glutamate release from the presynaptic terminal.

While these activities of PAF models appear to be independent of changes in gene expression, they nevertheless require glutamate release from the presynaptic axonal terminal to result in the accumulation of PAF in the postsynaptic dendritic terminal and cell body. Thus, under conditions of prolonged stimulation or massive glutamate release, there is the potential to greatly enhance PAF accumulation in these cellular structures, and hence the potential for activation of the intracellular PAF receptor and induction of gene expression (Figure 3).

CONCLUSIONS

PAF, produced rapidly after brain injury, is involved in the delayed (~3–4h post-injury) and prolonged (24h post-injury) up-regulation of COX-2 expression and synthesis of prostaglandins. In seizures and ischaemia this correlates with vulnerable parts of the brain e.g. hippocampus. COX-2 expression is inhibited by the intracellular PAF antagonist BN 50730 and by the glucocorticoid dexamethasone in ECS and KA-induced seizures. Inhibition by the PAF antagonist and dexamethasone correlates with inhibition of brain oedema in a cryogenically-induced vasogenic oedema model. Protection by PAF antagonists in cerebral ischaemia also correlates with release of lipid second messengers.

What aspects of COX-2 activation, if any, cause pathophysiological effects? Increased production of PGE_2 has been suggested to be neuroprotective in some models. However, the burst of oxidative metabolism that occurs with reperfusion following ischaemia is accompanied by an increased generation of reactive oxygen species and other free radicals. The peroxidase activity of PG synthase has been shown to catalyse the generation of reactive oxygen species from a number of substrates. Thus, overexpression of COX-2 could contribute to oxidative stress.

The identification of distinct pathways by which COX-2 expression can be modulated, and the development of novel COX-2 enzyme inhibitors, provides us with several new strategies for targeting inducible cyclooxygenase activation in neuroprotection (Figure 4). COX-2 overexpression during brain injury may be an important link between the acute effects of neuronal and/or cerebrovascular damage, and long-term deficits in brain function (Figure 5). Further research in this field holds promise for the treatment of brain damage and neurodegenerative diseases.

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References

1. Bazan NG. Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. Biochim Biophys Acta. 1970;218:1–10.
2. Nicotera P, Bellomo G, Orrenius S. Calcium-mediated mechanisms in chemically induced cell death. Annu Rev Pharmacol Toxicol. 1992;32:449–70.
3. Siesjö BK, Ingvar M, Westerberg Z. The influence of bicuculline-induced seizures on free fatty acid concentration in cerebral cortex, hippocampus and cerebellum. J Neurochem. 1992;39: 796–802.
4. Yoshida S, Harik S, Busto R, Santiso M, Martinez E, Ginsberg MD. Free fatty acids and energy metabolites in ischemic cerebral cortex with noradrenaline depletion. J Neurochem. 1984;42: 711–7.

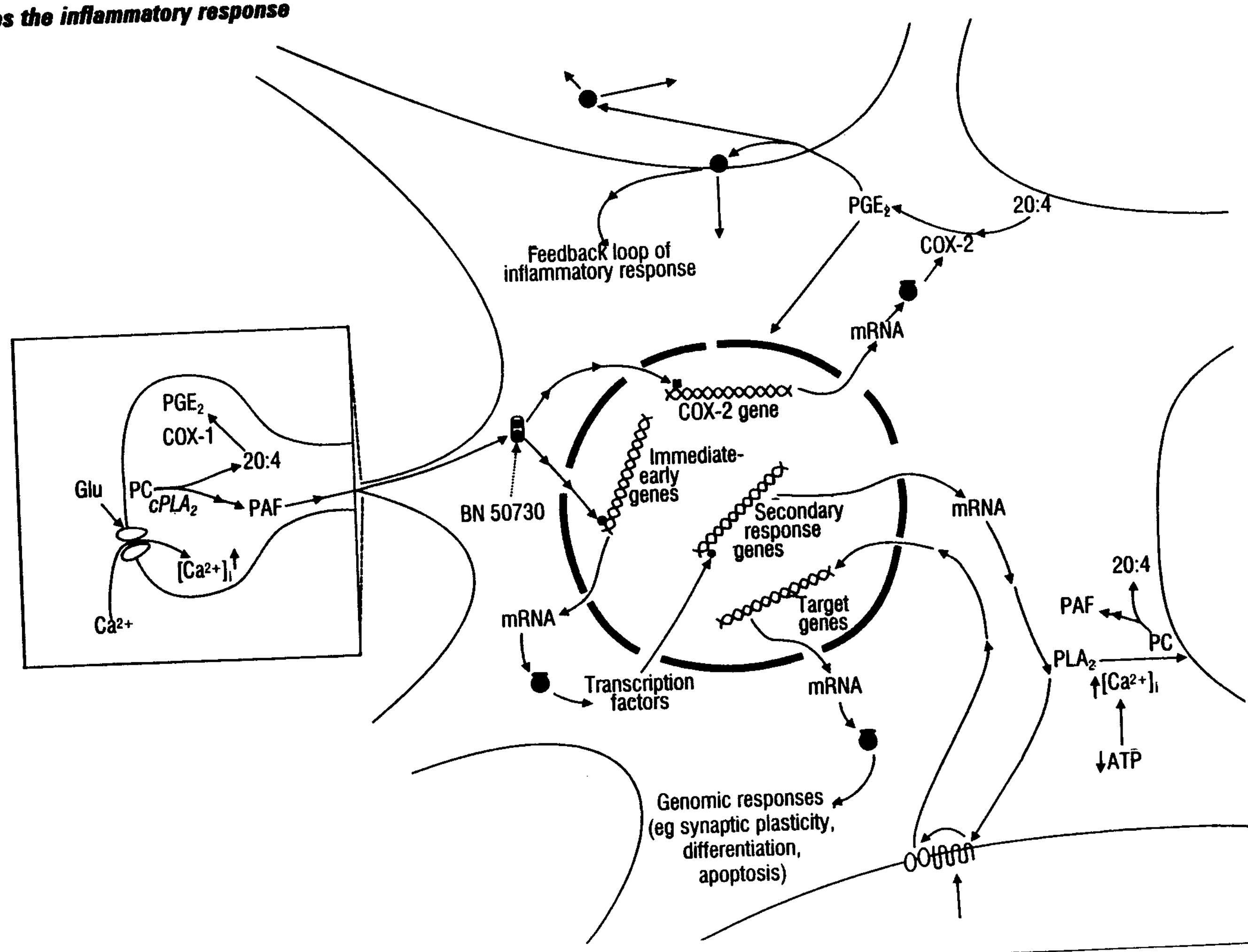

PGE$_2$
COX-1
20:4
Glu
PC
cPLA$_2$
PAF
[Ca^{2+}]$_i$
Ca^{2+}
BN 50730
Feedback loop of inflammatory response
PGE$_2$
20:4
COX-2
mRNA
COX-2 gene
Immediate-early genes
Secondary response genes
Target genes
mRNA
Transcription factors
mRNA
mRNA
Genomic responses (eg synaptic plasticity, differentiation, apoptosis)
20:4
PAF
PC
PLA$_2$
[Ca^{2+}]$_i$
ATP

Figure 3 *Coupling between short term neuronal signalling signals and longer-term changes in cellular physiology. Receptor-mediated synthesis of PAF in the postsynaptic terminal or cell body leads to activation of the intracellular PAF receptor. Through signalling mechanisms involving MAP and possibly other kinases, endogenous nuclear transcription factors are activated and promote the expression of immediate early genes (IEG). IEG transcription factors modulate the transcription of other genes, the expression of which can in turn lead to the expression of further sets of genes. These gene cascades are a mechanism by which expression of a small number of IEG can be amplified into profound long term changes in neuronal phenotype. Also shown is COX-2 (PGHS$_2$) which may be activated by distinct transcription factors. COX-2 expression augments prostaglandin synthesis and promotes the inflammatory response*

***Figure 4** Sites of regulation of PAF-induced COX-2 expression and prostaglandin synthesis. PLA_2 activation generates lyso-PAF, which is then transacetylated to form PAF. PAF has a short biological half-life. It is quickly deacetylated by PAF acetylhydrolase and only under stimulated conditions does sufficient PAF accumulate to activate the intracellular PAF receptor, the target for the PAF antagonist BN 50730. Receptor activation triggers a series of events which allows one or more transcription factors to bind to the 'PAF-responsive' domain on the COX-2 gene promoter. Glucocorticoids, bound to their nuclear receptor, may inhibit COX-2 gene induction by sterically hindering binding of the PAF-induced transcription factor. Immediate early gene mRNAs are inherently unstable, containing 3' sequences which direct their rapid degradation. COX-2 induction is longer-lived than, for instance, induction of transcription factor immediate-early genes such as c-fos; nevertheless, post-transcriptional regulation of COX-2 expression via proteins which bind to the mRNA is being investigated. Finally, the development of cyclooxygenase inhibitors specific for the inducible enzyme enables this pool of prostaglandin synthesis to be targeted in experimental and therapeutic approaches*

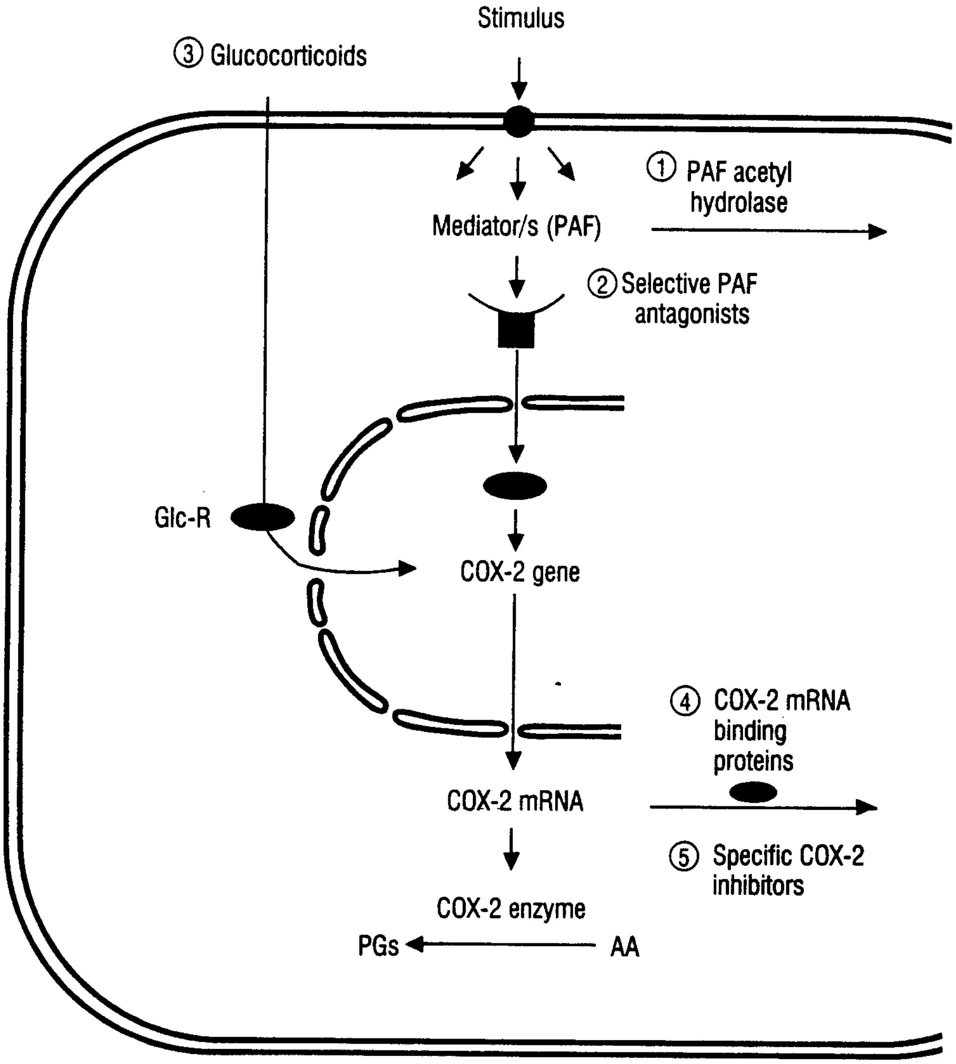
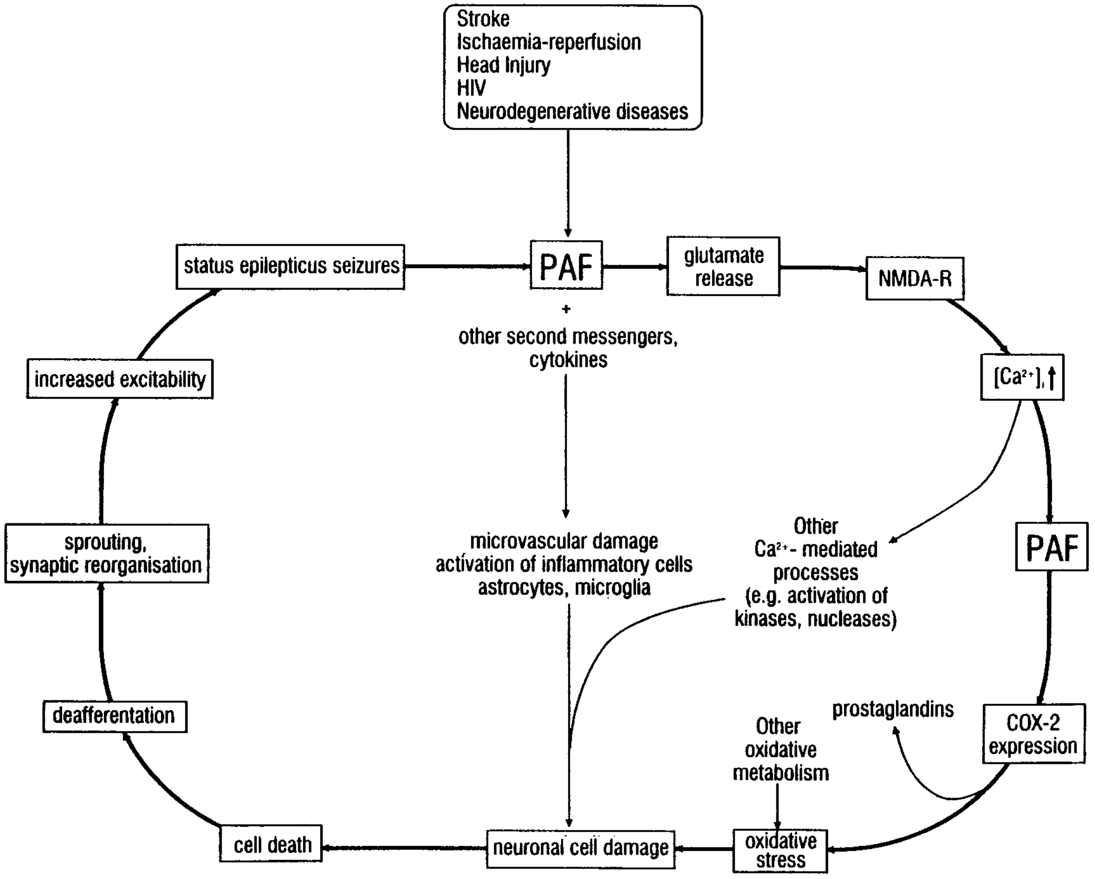

Stimulus
③ Glucocorticoids
① PAF acetyl hydrolase
Mediator/s (PAF)
② Selective PAF antagonists
Glc-R
COX-2 gene
④ COX-2 mRNA binding proteins
COX-2 mRNA
⑤ Specific COX-2 inhibitors
COX-2 enzyme
PGs
AA

Figure 5 ***PAF, COX-2 and the vicious cycle of brain pathology. Many factors contribute to the acute and long term effects of brain injury and neurodegenerative diseases. The aim of this figure is to pick out the strand in these many complex processes that involves PAF and COX-2 activation, and to show that therapeutic approaches involving these steps can potentially have profound effects not only on the immediate effects of neuronal cell damage and death, but also in the delayed neurological deficits such as aberrant synaptic plasticity and epileptogenesis which lead to a spiral in the normal functioning of the brain***


Stroke
Ischaemia-reperfusion
Head Injury
HIV
Neurodegenerative diseases
status epilepticus seizures
PAF
glutamate release
NMDA-R
[Ca²⁺]ᵢ↑
+
other second messengers, cytokines
increased excitability
sprouting, synaptic reorganisation
microvascular damage
activation of inflammatory cells
astrocytes, microglia
Other Ca²⁺- mediated processes (e.g. activation of kinases, nucleases)
PAF
deafferentation
prostaglandins
Other oxidative metabolism
COX-2 expression
cell death
neuronal cell damage
oxidative stress

5. Birkle DL, Bazan NG. Effect of bicuculline-induced status epilepticus on prostaglandins and hydroxyeicosatetraenoic acids in rat brain subcellular fractions. J Neurochem. 1987;48:1768–78.
6. Huang SF-L, Sun GY. Acidic phospholipids, diacylglycerols, and free fatty acids in gerbil brain: a comparison of ischemic changes resulting from carotid ligation and decapitation. J Neurosci Res. 1987;17:162–7.
7. Bazan NG. Arachidonic acid (AA) in the modulation of excitable membrane function and at the onset of brain damage. Ann NY Acad Sci. 1989;559:1–16.
8. Katsura K, Rodriguez de Turco EB, Folbergrova J, Bazan NG, Siesjö BK. Coupling among energy failure, loss of ion homeostasis, and phospholipase A2 and C activation during ischemia. J Neurochem. 1993;61:1677–84.
9. Pediconi MF, Rodriguez de Turco EB. Free fatty acid content and release kinetics as manifestations of cerebral lateralization in mouse brain. J Neurochem. 1984;43:1–7.
10. Yasuda H, Kishiro K, Izumi N, Nakanishi M. Biphasic liberation of arachidonic and stearic acids during cerebral ischemia. J Neurochem. 1985;45:168–72.
11. Katayama Y, Kawamata T, Maeda T, Ishikdawa K, Tsubokawa T. Inhibition of the early phase of free fatty acid liberation during cerebral ischemia by excitatory amino acid antagonist administered by microdialysis. Brain Res. 1994;635:331–4.
12. Farooqui AA, Hirashima Y, Horrocks LA. Brain phospholipases and their role in signal transduction. Adv Exp Med Biol. 1992;318:11–25.
13. Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science. 1992;258:607–14.
14. Shohami E, Shapira Y, Reisfeld N, Yedgar S. Brain phospholipase A_2 is activated after experimental closed head injury in the rat. J Neurochem. 1989;53:1541–6.
15. Rordorf G, Uemura Y, Bonventre JV. Characterization of phospholipase A2 (PLA2) activity in gerbil brain: enhanced activities of cytosolic, mitochondrial, and microsomal forms after ischemia and reperfusion. J Neurosci. 1991;11:1829–36.
16. Bonventre JV, Koroshetz WJ. Phospholipase A_2 (PLA_2) activity in gerbil brain: characterization of cytosolic and membrane-associated forms and effects of ischemia and reperfusion on enzymatic activity. J Lipid Mediat. 1993;6:457–71.
17. Aveldaño MI, Bazan NG. Rapid production of diacylglycerols enriched in 20:4 and stearate during early brain ischemia. J Neurochem. 1975;25:919–20.
18. Ikeda M, Yoshida S, Busto R, Santiso M, Ginsberg MD. Polyphosphoinositides as a probable source of brain free fatty acids accumulated at the onset of ischemia. J Neurochem. 1986;47: 123–32.
19. Reddy TS, Bazan NG. Arachidonic acid, stearic acid and diacylglycerol accumulation correlates with the loss of phosphatidylinositol 4,5-bisphosphate in cerebrum 2 seconds after electroconvulsive shock. Complete reversion of changes 5 minutes after stimulation. J Neurosci Res. 1987;18:449–55.
20. Rodriguez de Turco EB, Bazan NG. Changes in free fatty acids and diglycerides in mouse brain at birth and during anoxia. J Neurochem. 1983;41:794–800.
21. Bazan NG, Allan G, Rodriguez de Turco EB. Role of phospholipase A_2 and membrane-derived lipid second messengers in excitable membrane function and transcriptional activation of genes: Implications in cerebral ischemia and neuronal excitability. Prog Brain Res. 1993;96:247–57.
22. Rodriguez de Turco EB, Droy-Lefaix MT, Bazan NG. Decreased electroconvulsive shock-induced diacylglycerols and free fatty acid accumulation in the rat brain by *Ginkgo biloba* extracts (EGB 761): Selective effect in hippocampus as compared with cerebral cortex. J Neurochem. 1993;61:1438–44.
23. Sun GY. Contributions to arachidonic acid release in mouse cerebrum by the phosphoinositide-phospholipase C and phospholipase A2 pathways. Adv Exp Med Biol. 1992;318:103–14.
24. Sun GY, Lu FL, Lin SE, Ko MR. Decapitation ischemia-induced release of free fatty acids in mouse brain. Relationship with diacylglycerols and lysophospholipids. Mol Chem Neuropathol. 1992;17:39–50.
25. Umemura A, Mabe H, Nagai H, Sugino F. Action of phospholipase A_2 and C on free fatty acid release during complete ischemia in rat cortex. Effect of phospholipase C inhibitor and *N*-methyl-D-aspartate antagonist. J Neurosurg. 1992;76:648–51.
26. Berridge MJ. Inositol trisphosphate and calcium signalling. Nature. 1993;361:315–25.
27. Dumuis A, Sebben M, Haynes J-P, Bockaert J. NMDA receptors activate the arachidonic acid cascade system in striatal neurons. Nature. 1988;336:68–70.

28. Lazarewicz JW, Wroblewski JT, Palmer ME, Costa E. Activation of *N*-methyl-D-aspartate-sensitive glutamate receptors stimulates arachidonic acid release in primary cultures of cerebellar granule cells. Neuropharmacology. 1988;27:765–9.
29. Pellerin L, Wolfe LS. Release of arachidonic acid by NMDA-receptor activation in the rat hippocampus. Neurochem Res. 1991;16:983–9.
30. Sanfeliu C, Hunt A, Patel AJ. Exposure to *N*-methyl-D-aspartate increases release of arachidonic acid in primary cultures of rat hippocampal neurons and not in astrocytes. Brain Res. 1990;256:241.
31. Yoshijara Y, Watanabe Y. Translocation of phospholipase A_2 from cytosol to membranes in rat brain induced by calcium ions. Biochem Biophys Res Commun. 1990;170:484–90.
32. Fujimori Y, Murakami M, Kim DK et al. Immunochemical detection of arachidonoyl-preferential phospholipase A2. J Biochem (Tokyo). 1992;111:54–60.
33. Kudo I, Murakami M, Hara S, Inoue K. Mammalian non-pancreatic phospholipases A2. Biochem Biophys Acta. 1993;1170:217–31.
34. Axelrod J, Burch RM, Jelsema CL. Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: Arachidonic acid and its metabolites as second messengers. Trends Neurochem Sci. 1988;11:117–23.
35. Jelsema CL, Axelrod J. Stimulation of phospholipase A_2 activity in bovine rod outer segment by the β gamma subunits of transducin and its inhibition by the α subunit. Proc Natl Acad Sci USA. 1987;84:3623–7.
36. Peitsch MC, Borner C, Tschopp J. Sequence similarity of phospholipase A2 activating protein and the G protein beta-subunits: a new concept of effector protein activation in signal transduction? Trends Biochem Sci. 1993;18:292–3.
37. Flower RJ. Lipocortin and the mechanism of action of the glucocorticoids. Br J Pharmacol. 1988;79:987–1015.
38. Strijbos PJLM, Tilders FJN, Carey F, Forder RA, Rothwell NJ. Localization of immunoreactive lipocortin-1 in the brain and pituitary gland of the rat. Brain Res. 1991;553:249–60.
39. Rothwell NJ, Relton JK. Involvement of interleukin-1 and lipocortin-1 in ischaemic brain damage. Cerebrovasc Brain Metab Rev. 1993;5:178–98.
40. Relton JK, Strijbos PJLM, O'Shaughnessey CT et al. Lipocortin-1 is an endogenous inhibitor of ischemic damage in the rat brain. J Exp Med. 1991;174:305–10.
41. Black MD, Carey F, Crosman AR, Relton JK, Rothwell N. Lipocortin-1 inhibits NMDA receptor-mediated neuronal damage in the striatum of the rat. Brain Res. 1992;585:135–40.
42. Shimizu T, Wolfe LS. Arachidonic acid cascade and signal transduction. J Neurochem. 1990;55: 1–15.
43. Volterra A, Trotti D, Cassutti P et al. A role for the arachidonic acid cascade in fast synaptic modulation: ion channels and transmitter uptake systems as target proteins. Adv Exp Med Biol. 1992;318:147–58.
44. Ordway RW, Singer JJ, Walsh JV Jr. Direct regulation of ion channels by fatty acids. Trends Neurosci. 1991;14:96.
45. Bazan NG. Involvement of arachidonic acid and platelet-activating factor in the response of the nervous system to ischemia and convulsions. In: Bazan NG, editor. Lipid Mediators in Ischemic Brain Damage and Experimental Epilepsy. New Trends in Lipid Mediators. Basel: Karger; 1990:241–52.
46. Yu ACH, Chan PH, Fishman RA. Effects of arachidonic acid on glutamate and gamma-aminobutyric acid uptake in primary culture of rat cerebral cortical astrocytes and neurons. J Neurochem. 1986;47:1181–9.
47. Barbour B, Szatkowski M, Ingledew N, Attwell D. Arachidonic acid induces a prolonged inhibition of glutamate uptake into glial cells. Nature. 1989;342:918–20.
48. Dorman RV, Hamm TF, Damron DS, Freeman EJ. Modulation of glutamate release from hippocampal mossy fiber nerve endings by arachidonic acid and eicosanoids. Adv Exp Med Biol. 1992;318:121–36.
49. Zhange L, Dorman RV. Synergistic potentiation of glutamate release by arachidonic acid and oleoyl-acetyl-glycerol. Brain Res Bull. 1993;32:437–41.
50. Domanska-Janik K. Unsaturated fatty acids induce inhibition of the A1-adenosine receptor in rat brain membranes. Neuro Report. 1992;4:451–53.
51. Miller B, Sarantis M, Traynelis SF, Attwell D. Potentiation of NMDA receptor currents by arachidonic acid. Nature. 1992;355:722–5.

52. Petrou S, Ordway RW, Singer JJ, Walsh JV Jr. A putative fatty acid-binding domain of the NMDA receptor. Trends Biochem Sci. 1993;18:41–2.
53. Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature. 1993;361:31–9.
54. Piomelli D. Eicosanoids in synaptic transmission. Crit Rev Neurobiol. 1994;8:65–83.
55. Gaudet RJ, Alam I, Levine L. Accumulation of cyclooxygenase products of arachidonic acid metabolism in gerbil brain during reperfusion after bilateral common carotid artery occlusion. J Neurochem. 1981;35:653.
56. Kempski O, Shohami E, von Lubitz D, Hallenbeck JM, Feuerstein G. Postischemic production of eicosanoids in gerbil stroke. Stroke. 1987;18:111–9.
57. Moskowitz MA, Kiwak KJ, Hekiman K, Levine L. Synthesis of compounds with properties of leukotrienes C_4 and D_4 in gerbil brains after ischemia and reperfusion. Science. 1984;224:886.
58. Dempsey RJ, row MW, Meyer K, Cowen DE, Tai HH. Development of cyclooxygenase and lipoxygenase metabolites of arachidonic acid after transient cerebral ischemia. J Neurosurg. 1986;64:118–24.
59. Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A_2 and prostacyclin. Pharmacol Rev. 1979;30:293–331.
60. Unterberg A, Wahl M, Hammerson F, Baethman A. Permeability and vasomotor response of cerebral vessels during exposure to arachidonic acids. Acta Neuropathol (Berl). 1987;73: 209–11.
61. Baba T, Black KL, Ikezaki K, Chen KN, Becker DP. Intracarotid infusion of leukotriene C4 selectively increases blood–brain barrier permeability after focal ischemia in rats. J Cerebr Blood Flow Metab. 1991;11:638–43.
62. Herschman HR. Regulation of prostaglandin synthase-1 and prostaglandin synthase-2. Cancer Metastatis Rev. 1994;13:241–56.
63. Vane JR, Botting RM. A better understanding of anti-inflammatory drugs based on isoforms of cyclooxygenase (COX-1 and COX-2). Adv Prost Thromb Leuk Res. 1995;23:41–8.
64. Kujubu DA, Fletcher BS, Varnum BC, Lim RW, Herschman HR. TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J Biol Chem. 1991;266:12866–72.
65. Xie W, Chipman JG, Robertson DL, Erikson RL, Simmons DL. Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc Natl Acad Sci USA. 1991;88:2692–6.
66. Yamagata K, Andreasson KI, Kaufmann WE, Barnes CA, Worley PF. Expression of a mitogen-inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids. Neuron. 1993;11:371–86.
67. Breder CD, Dewitt D, Kraig RP. Characterization of inducible cyclooxygenase in rat brain. J Comp Neurol. 1995;355:296–315.
68. Henson PM. Release of vasoactive amines from rabbit platelets induced by antiplatelet antibody in the presence and absence of complement. J Immunol. 1970;104:934.
69. Benveniste J, Henson PM, Cochrane CG. Leukocyte-dependent histamine release from rabbit platelets. The role of IgE, basophils, and platelet-activating factor. J Exp Med. 1972;136: 1356–77.
70. Braquet P, Touqui L, Shen TY, Vargaftig BB. Perspectives in platelet-activating factor research. Pharmacol Rev. 1987;39:97–145.
71. Prescott SM, Zimmerman GA, McIntyre TM. Platelet-activating factor. J Biol Chem. 1990; 265:17381–4.
72. Bussolino F, Soldi R, Arese M, Jaranowska A, Sogos V, Gremo F. Multiple roles of platelet-activating factor in the nervous system. Neurochem Int. 1995;26:425 33.
73. Kumar R, Harvey S, Kester N, Hanahan D, Olsen M. Production and effects of platelet-activating factor in the rat brain. Biochim Biophys Acta. 1988;963:375–83.
74. Tiberghein C, Laurent L, Junier MP, Dray F. A competitive receptor binding assay for platelet-activating factor (PAF): quantification of PAF in rat brain. J Lipid Mediat. 1991;3:249–66.
75. Bussolino F, Torelli S, Stefanini E, Gremo F. Platelet-activating factor production occurs through stimulation of cholinergic and dopaminergic receptors in the chick retina. J Lipid Mediat. 1989;1:283–8.
76. Panetta T, Marcheselli VL, Braquet P, Spinnewyn B, Bazan NG. Effects of a platelet-activating

factor antagonist (BN 52021) on free fatty acids, diacylglycerols, polyphosphoinositides and blood flow in the gerbil brain. Inhibition of ischemia-reperfusion induced cerebral injury. Biochem Biophys Res Commun. 1987;149:580–7.

77. Sogos V, Bussolino F, Pilia E, Torelli S, Gremo F. Acetylcholine-induced production of platelet-activating factor by human fetal brain cells in culture. J Neurosci Res. 1990;27:706–11.
78. Goracci G, Francesangeli E. Properties of PAF-synthesizing phosphocholinetransferase and evidence for lysoPAF acetyltransferase activity in brain. Lipids. 1991;26:986–91.
79. Blank ML, Smith ZL, Fitzgerald V, Snyder F. The CoA-independent transacylase in PAF biosynthesis: tissue distribution and molecular species selectivity. Biochim Biophys Acta. 1995; 1254:295–301.
80. Lee T-C, Malone B, Snyder F. Formation of 1-alkyl-2-acetyl-sn-glycerols via the de novo biosynthetic pathway for platelet-activating factor. J Biol Chem. 1988;263:1755–60.
81. Deshpande JK, Siesjö BK, Wieloch T. Calcium accumulation and neuronal damage in the rat hippocampus following cerebral ischemia. J Cerebr Blood Flow Metab. 1987;7:89–95.
82. Francescangeli E, Goracci G. The de novo biosynthesis of platelet-activating factor in rat brain. Biochem Biophys Res Commun. 1989;161:107–12.
83. Marcheselli VL, Rossowska M, Domingo MT, Braquet P, Bazan NG. Distinct platelet-activating factor binding sites in synaptic endings and in intracellular membranes of rat cerebral cortex. J Biol Chem. 1990;265:9140–5.
84. Honda Z, Nakamura M, Miki I et al. Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. Nature. 1991;394:342–6.
85. Kunz D, Gerard NP, Gerard C. The human leukocyte PAF receptor. cDNA cloning, cell surface expression and construction of a novel epitope-bearing analog. J Biol Chem. 1992;267:9101–6.
86. Sugimoto T, Tsuchimochi H, McGregor CG, Mutoh H, Shimizu T, Kurachi Y. Molecular cloning and characterization of the platelet-activating factor receptor from human heart. Biochem Biophys Res Commun. 1992;189:617–24.
87. Sasaki Y, Usui T, Tanaka I et al. Cloning and expression of a cDNA for rat prostacyclin receptor. Biochim Biophys Acta. 1994;1224:601–5.
88. Mutoh H, Bito H, Minami M et al. Two different promoters direct expression of two distinct forms of mRNA of human platelet-activating factor receptor. FEBS Lett. 1993;322:129–34.
89. Bito H, Nakamura M, Honda Z et al. Platelet-activating factor (PAF) receptor in rat brain: PAF mobilizes intracellular Ca^{2+} in hippocampal neurons. Neuron. 1992;9:285–94.
90. Ali H, Richardson RM, Tomhave ED, DuBose RA, Haribabu B, Snyderman R. Regulation of stably transfected platelet activating factor receptor in RBL-2H3 cells. Role of multiple G proteins and receptor phosphorylation. J Biol Chem. 1994;269:24557–63.
91. Honda Z, Takano T, Hirose N et al. Gq pathway desensitizes chemotactic receptor-induced calcium signaling via inositol trisphosphate receptor down-regulation. J Biol Chem. 1995; 270:4840–4.
92. Honda Z, Takano T, Gotoh Y, Nishida E, Ito K, Shimizu T. Transfected platelet-activating factor receptor activates mitogen-activated protein (MAP) kinase and MAP kinase in Chinese hamster ovary cells. J Biol Chem. 1994;269:2307–15.
93. Kornecki E, Ehrlich YH. Neuroregulatory and neuropathological actions of the ether-phospholipid platelet-activating factor. Science. 1988;240:1792–4.
94. Kuruvilla A, Pielop C, Shearer WT. Platelet-activating factor induces the tyrosine phosphorylation and activation of phospholipase C-γ1, fyn and lyn kinases, and phosphatidylinositol 3-kinase in a human B cell line. J Immunol. 1994;153:5433–42.
95. Squinto SP, Block AL, Braquet P, Bazan NG. Platelet-activating factor stimulates a Fos/Jun/AP-1 transcriptional signaling system in human neuroblastoma cells. J Neurosci Res. 1989;24: 558–66.
96. Pan Z, Kravchenko VV, Ye RD. Platelet activating factor stimulates transcription of the heparin-binding epidermal growth factor-like growth factor in monocytes. Correlation with an increased kappa B binding activity. J Biol Chem. 1995;270:7787–90.
97. Mazer B, Domenico J, Sawami H, Gelfand EW. Platelet-activating factor induces an increase in intracellular calcium and expression of regularity genes in human B lymphoblastoma cells. J Immunol. 1994;146:1914–20.
98. Smith CS, Shearer WT. Activation of NF-kappa B and immunoglobulin expression in response to platelet-activating factor in a human B cell line. Cell Immunol. 1994;155:292–303.

99. Dell'Albani P, Condorelli DF, Mudo G, Amico C, Bindoni M, Belluardo N. Platelet-activating factor and its methoxy analogue ET-18-OCH3 stimulate immediate-early gene expression in rat astroglial cultures. Neurochem Int. 1993;22:567–74.
100. Tripathi Y, Kandala J, Guntaka R, Lim R, Shukla S. Platelet-activating factor induces expression of early response genes c-*fos* and TIS-1 in human epidermoid carcinoma-432 cells. Life Sci. 1991;49:1761–7.
101. Mutoh H, Ishii S, Izumi T, Kato S, Shimizu T. Platelet-activating factor (PAF) positively autoregulates the expression of human PAF receptor transcript 1 (leukocyte-type) through NF-kappa B. Biochem Biophys Res Commun. 1994;205:1137–42.
102. Shirasaki H, Adcock IM, Kwon OJ, Nishikawa M, Mak JC, Barnes PJ. Agonist-induced upregulation of platelet-activating factor receptor messenger RNA in human monocytes. Eur J Pharmacol. 1994;268:263–6.
103. Yokoyama C, Tanabe T. Cloning of the human gene encoding prostaglandin endoperoxide synthase and primary structure of the enzyme. Biochem Biophys Res Commun. 1989;165: 888–94.
104. Kraemer SA, Meade EA, DeWitt DL. Prostaglandin endoperoxide synthase gene structure: identification of the transcriptional start site and 5′-flanking regulatory sequences. Arch Biochem Biophys. 1992;293:391–400.
105. Fletcher BS, Kujubu DA, Perrin DM, Herschman HR. Structure of the mitogen-inducible TIS10 gene and demonstration that the encode protein is a functional prostaglandin synthase. J Biol Chem. 1992;267:4338–44.
106. Xie W, Merril JR, Bradshaw WS, Simmons DL. Structural determination and promoter analysis of the chicken mitogen-inducible prostaglandin G/H synthase gene and genetic mapping of the murine homolog. Arch Biochem Biophys. 1994;300:247–52.
107. Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T. Structure of the human cyclo-oxygenase-2 gene. Biochem J. 1994;302:723–7.
108. Tazawa R, Xu XM, Wu KK, Wang LH. Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. Biochem Biophys Res Commun. 1994;203:190–9.
109. Sirois J, Richards JS. Transcriptional regulation of the rat prostaglandin endoperoxide synthase 2 gene in granulosa cells. Evidence for the role of a cis-acting C/EBP beta promoter element. J Biol Chem. 1993;268:21913–8.
110. Bazan NG, Fletcher BS, Herschman HR, Mukherjee PK. Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene. Proc Natl Acad Sci USA. 1994;91:5252–6.
111. Babb TL, Leib JP, Brown WJ, Pretorius J, Crandell PH. Distribution of pyramidal cell density and hyperexcitability in the epileptic human hippocampal formation. Epilepsia. 1984;25:71–9.
112. Bazan NG, Politi E, Rodriguez de Turco EB. Endogenous pools of arachidonic acid-enriched membrane lipids in cryogenic brain injury. In: Go KG, Baethmann A, editors. Recent Progress in the Study and Therapy of Brain Edema. New York: Plenum; 1984:203–12.
113. Hurst JS, Bazan HE. The platelet-activating factor precursor of the injured cornea is selectively implicated in arachidonate and eicosanoid release. Curr Eye Res. 1993;12:655–63.
114. Bazan HEP, Tao Y, Bazan NG. Platelet activating factor induces collagenase expression in corneal epithelium cells. Proc Natl Acad Sci USA. 1993;90:8672–82.
115. Tao Y, Bazan HEP, Bazan NG. Platelet-activating factor (PAF) induces expression of the urokinase type, but not the tissue type plasminogen activator in the corneal epithelium. Invest Ophthalmol Vis Sci. 1994;35(Suppl.):S1457.
116. Tao Y, Bazan HEP, Bazan NG. Platelet-activating factor (PAF) increases prostaglandin synthase-2 (COX-2) gene expression in corneal epithelium. Invest Ophthalmol Vis Sci. 1995: 36:S573.
117. Tao Y, Bazan HEP, Bazan NG. Platelet-activating factor induces the metalloproteinase-1 and -9 but not -2 or -3, in the corneal epithelium. Invest Ophthalmol Vis Sci. 1995;36:345–54.
118. Fini ME, Girard MT, Matsubara M. Collagenolytic/gelatinolytic enzymes in cornea wound healing. Acta Ophthalmol. 1992;202(Suppl.):26–33.
119. Clohisy JC, Connolly TJ, Bergman KD, Quinn CO, Partridge NC. Prostanoid-induced expression of matrix metalloproteinase-1 mRNA in rat osteosarcoma cells. Endocrinology. 1994; 135:1447–54.

120. Mertz PM, DeWitt DL, Stetler-Stevenson WG, Wahl LM. Interleukin 10 suppression of monocyte prostaglandin H synthase-2. Mechanisms of inhibition of prostaglandin-dependent matrix metalloproteinase production. J Biol Chem. 1994;269:21322–29.
121. Ito A, Nose T, Takahashi S, Mori Y. Cyclooxygenase inhibitors augment the production of pro-matrix metalloproteinase-9 (progelatinase B) in rabbit articular chondrocytes. FEBS Lett. 1995;360:75–9.
122. Salvatori R, Guidon PT Jr, Rapuano BE, Bockman RS. Prostaglandin E1 inhibits collagenase gene expression in rabbit synoviocytes and human fibroblasts. Endocrinology. 1992;131:21–8.
123. Clark GD, Happel LT, Zorumski CF, Bazan NG. Enhancement of hippocampal excitatory synaptic transmission by platelet-activating factor. Neuron. 1992;9:1211–6.
124. Kato K, Clark GD, Bazan NG, Zorumski CF. Platelet-activating factor as a potential retrograde messenger in CA1 hippocampal long-term potentiation. Nature. 1994;367:179–82.
125. Izquierdo I, Fin C, Schmitz PK et al. Memory enhancement by intrahippocampal, intra-amygdala, or intraentorhinal infusion of platelet-activating factor measured in an inhibitory avoidance task. Proc Natl Acad Sci USA. 1995;92:5047–51.
126. Jerusalinsky D, Quillfeldt JA, Walz R, Da Silva R, Medina JH, Izquierdo I. Post-training intrahippocampal infusion of protein kinase C inhibitors causes amnesia in rats. Behav Neurol Biol. 1994;61:107–9.
127. Korol DL, Abel TW, Church LT, Barnes CA, McNaughton BL. Hippocampal synaptic enhancement and spatial learning in the Morris swim task. Hippocampus. 1993;3:127–32.

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Sustained Induction of Prostaglandin Endoperoxide Synthase-2 by Seizures in Hippocampus

INHIBITION BY A PLATELET-ACTIVATING FACTOR ANTAGONIST*

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Prostaglandin G/H synthase-2 and *zif*-268 mRNA expression is transiently induced in rat brain by kainic acid (KA)-induced seizures and by a single electroconvulsive shock. Induction of both genes by KA shows neuroanatomical specificity in the order hippocampus > cerebral cortex > striatum > brain stem > cerebellum. Nuclear run-on and Western blotting shows that both genes are transcriptionally activated, and that kainic acid up-regulation of prostaglandin G/H synthase-2 mRNA expression in hippocampus matches increased protein levels. Whereas the magnitude of hippocampal *zif*-268 mRNA induction is similar in both seizure models, peak induction of prostaglandin G/H synthase-2 mRNA is 7-fold greater in the kainic acid model than in the electroconvulsive shock model and is much more prolonged. Pretreatment of animals by intracerebroventricular injection with the intracellular platelet-activating factor receptor antagonist BN 50730 strongly attenuates kainic acid and electroconvulsive shock induction of prostaglandin G/H synthase-2 expression. The drug partially inhibits electroconvulsive shock induction of *zif*-268, but is relatively ineffective against kainic acid-induced *zif*-268 expression. Seizure-induced expression of both genes involves platelet-activating factor, but the mechanisms of induction must be otherwise distinct. The selectively elevated induction of hippocampal prostaglandin G/H synthase-2 by kainic acid correlates with a neuroanatomical region in which the agonist induces neuronal damage.

Seizure activity in the brain initiates complex pathways of signal transduction and cell-to-cell communication. Whereas a single seizure usually has little or no long-term effects on the brain, repeated and uncontrolled seizures can cause delayed neuronal death and synaptic reorganization. Activation of phospholipases and accumulation of bioactive lipids takes place early after seizures (1) primarily in synaptic endings (2). Arachidonic acid and its oxygenated metabolites and platelet-activating factor are important classes of bioactive lipids generated during seizure activity (3, 4) because they are known to have modulatory effects on synaptic transmission and neuronal plasticity (5–7). Additionally, PAF[1] is also an activator of receptor-mediated immediate early gene expression in the brain and neuronal cells (8, 9). Thus, excitable membrane-derived bioactive lipids may have both acute and long-term effects on neuronal activity.

PAF exerts its biological actions through a rhodopsin-type receptor (10). In neurons, this receptor mediates the presynaptic effects of PAF on excitatory neurotransmitter release (6), long-term potentiation (7, 11), and memory formation (12, 13). A high affinity PAF binding site has been found in microsomal membranes isolated from hippocampus and cerebral cortex (9, 14). This site has distinct ligand binding kinetics and sensitivity to PAF receptor antagonists and may represent either an intracellular form of the plasma membrane receptor or a novel PAF receptor subtype. The intracellular PAF-binding site may be involved in the induction of immediate-early transcription factors in the hippocampus during electrically induced seizures (9). Additionally, PAF activates the expression of transfected prostaglandin G/H synthase-2 (PGS-2, cyclooxygenase, COX-2) promoter constructs, and this effect is blocked by a PAF antagonist (BN 50730) selective for the intracellular site (15). PGS catalyzes the first committed step in the conversion of arachidonic acid to prostaglandins and thromboxanes. The PGS-2 isoform is normally expressed in most tissues only as a rapid and transient response to mitogenic and inflammatory stimuli, and is encoded by an immediate-early gene (16). Brain, however, is one of the few anatomical sites in which a basal level of PGS-2 expression has been demonstrated (17). This expression is localized to selected groups of neurons and is regulated by synaptic activity and, more specifically, by activation of the *N*-methyl-D-aspartate class of glutamate receptors (17, 18).

In this study, we tested the hypothesis that PAF is involved in the seizure-induced accumulation of PGS-2 in the brain and is thus linked to the metabolism of free arachidonic acid generated during seizures. We present evidence that PGS-2 transcriptional activity and protein expression is up-regulated in a single electrically-induced seizure (ECS) and in multiple, kainic acid (KA)-induced seizures, and that PAF is involved in this mechanism. In addition, we show that induction of PGS-2, but not the transcription factor immediate-early gene *zif*-268, is more sensitive to the severe KA-induced seizures than ECS in a brain region, the hippocampus, selectively vulnerable to damage in this model. Partial accounts of these results have appeared in abstract form (19).

EXPERIMENTAL PROCEDURES

Animals—Rats (Albino male Wistar, 250–320 g) from Charles River Breeding Laboratories, Wilmington, MA, were housed in the LSUMC

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[1] The abbreviations used are: PAF, platelet-activating factor; PGS-2, prostaglandin G/H synthase-2, cyclooxygenase, EC 1.14.99.1, COX-2, TIS-10; PGS-1, prostaglandin G/H synthase-1; KA, kainic acid; ECS, electroconvulsive shock; icv, intracerebroventricular; ip, intraperitoneal; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

animal facility for at least 24 h before experiments were performed.

Kainic Acid-induced Seizures—In one series of experiments, rats received 10 mg/kg KA in saline by ip injection (150 μl). Controls received saline alone. At 0, 1, 2, 3, 6, 12, and 24 h after injection, animals were anesthetized with ether and decapitated. In a second series of experiments, rats were anesthetized with ether and received intracerebroventricular (icv) injections of the PAF receptor antagonist BN 50730 in dimethyl sulfoxide (Me$_2$SO) (2 μl/ventricle, 100 μg/kg) or Me$_2$SO alone as a control. 15 min later, KA was injected ip (6 mg/kg). Animals were anesthetized with ether and decapitated at 2 or 6 h after KA treatment.

Single Electroconvulsive Shock (ECS)—Two platinum needle electrodes were implanted 1 cm apart under the scalp of the rats. A train of pulses was delivered at 100 V, 0.5-ms pulse duration, frequency 150 pulses/s, train duration 750 ms. Rats were then killed by ether anesthesia followed by decapitation at 0, 1, 2, 6, and 12 h after ECS. Other rats under ether anesthesia were pretreated with BN 50730 in Me$_2$SO (2 μl/ventricle, 100 μg/kg, icv) 15 min before ECS. Animals were then killed by ether anesthesia followed by decapitation at 0, 1, 2, 6, and 12 h after ECS.

Sampling of Brain Regions—After decapitation, the brain was rapidly excised, and hippocampus, brain cortex, brain stem, striatum, and cerebellum were rapidly dissected on a ice-cold dissection board. Tissues were homogenized with a Polytron type homogenizer in different buffers according to the assays to be performed. For RNA extraction, buffer A (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% *n*-lauroyl sarcosine, and 0.1 M β-mercaptoethanol, pH 7.0, 4 °C) was used. For Western blots, buffer B was used (10 mM Tris-HCl, 10 mM EDTA, 5 mM EGTA, 1% Triton X-100, pH 7.4, 4 °C). For nuclear isolation, buffer C was used (0.32 M sucrose, 1 mM MgCl$_2$, pH 7.4, 4 °C).

RNA Extraction and Northern Blot Analysis—Total cellular RNA was isolated using the guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (20). Gel electrophoresis of RNA (5 μg/lane) was performed under denaturing conditions on a 1.2% agarose gel. RNA was transferred to Hybond-N nylon membranes (Amersham International) and were hybridized at 42 °C with ^{32}P-labeled DNA probes for mouse *zif*-268 and PGS-2 (TIS-8 and TIS-10, gifts from Dr. Harvey Herschman, Dept. of Biological Chemistry, UCLA), COX-1, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, American Type Culture Collection, accession 57090). Signals were detected by x-ray film autoradiography or phosphorimaging (GS-250 Molecular Imager, Bio-Rad).

Nuclear Run-on Transcription—Nuclear fractions were isolated from hippocampus using differential centrifugation (21), frozen in liquid nitrogen, and stored at −20 °C until use. Samples were resuspended in 100 μl of 2× reaction buffer containing 0.1 M each of ATP, CTP, and GTP, and 0.1 M dithiothreitol. The reaction was started by the addition of 12.5 μl of [^{32}P]UTP (800 Ci/mmol, 40 mCi/ml) and incubated for 30 min at 30 °C with agitation. DNase I solution (0.6 ml) was added, then incubated for 5 min at 30 °C with agitation. Each sample received 200 μl of buffer (0.5 M Tris-Cl, pH 7.4, 0.125 M EDTA, 5% SDS) and 10 μl of proteinase K solution and were incubated at 40 °C for 30 min, and 10 μl of 10 mg/ml tRNA carrier was added. Samples were extracted with 1 ml of buffered phenol:chloroform:isoamyl alcohol (25:24:1), mixed carefully, and centrifuged for 10 min, 6000 × *g* at 4 °C. The aqueous phase was retained, and the organic phase was re-extracted with 2 ml of TE buffer, pH 8.0. RNA was precipitated from the combined aqueous phases by addition of 3 ml of 10% trichloroacetic acid in 60 mM sodium pyrophosphate and incubation on ice for 30 min. Precipitates were filtered onto Millipore type HA (0.45 μm) filters, washed 3 times with 10 ml of ice-cold 5% trichloroacetic acid in 30 mM sodium pyrophosphate, and loaded into scintillation vials. DNase I buffer (1.5 ml) and DNase I (37.5 μl, 1 mg/ml) were added, and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of EDTA (0.5 M, 45 μl) and SDS (20%, 68 μl). After heating at 65 °C for 10 min to elute the RNA, eluates were removed and collected. 0.75 ml of 10 mM Tris-HCl, 5 mM EDTA, 1% SDS (pH 7.5) was added to the eluates, and the mixture was incubated at 65 °C for 10 min. After adding proteinase K (4.5 μl, 20 mg/ml) and incubating at 37 °C for 30 min, the mixture was extracted with 3 ml of buffered phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was retained and mixed with 0.75 ml of 1 M NaOH. After incubation on ice for 10 min, the hydrolysis was stopped by neutralization with 1.5 ml of 1 M HEPES. RNA was precipitated with 0.53 ml of 5 M sodium acetate and 14.5 ml of 95% ethanol. After overnight storage at −20 °C and centrifugation at 6000 × *g*, 10 min, 4 °C, RNA was dried under vacuum and resuspended in 100 μl of 10 mM TES (10 mM Tris, pH 7.4, 0.2% SDS, 10 mM EDTA). Total radioactivity was calculated by counting 2-μl aliquots, and each sample was adjusted to 2.5 × 10^6 dpm in 300 μl. Hybridizations were carried out in 7-ml scintillation vials. A slot-blot filter strip with immobilized target plasmid cDNAs was incubated at 42 °C with 300 μl of RNA solution and 300 μl of 10 mM Tris-HCl (pH 7.4), 0.2% SDS, 10 mM EDTA, 600 mM NaCl in a rotary incubator. Filters were washed for 1 h in 2 × SSPE, 0.1% SDS at 65 °C and exposed to PhosphorImager plates or x-ray film.

Western Blotting—Tissue samples were kept on ice after dissection until homogenization. Hippocampal or cortical tissues were homogenized in a 2-ml glass/glass homogenizer with 0.5 ml of buffer B (described above). Protease inhibitors were added just before use (0.1 M phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitory unit/ml aprotinin, and 0.1 M leupeptin) at room temperature. Homogenates were sonicated for 30 s. Protein concentration was measured and adjusted to 2 μg/μl with buffer B. An equal volume of 2 × concentrated electrophoresis sample buffer (250 mM Tris-HCl, pH 6.5, 2% SDS, 10% glycerol, 0.006% bromphenol blue, and 10 μl/ml β-mercaptoethanol) was added to each sample, which were then boiled for 3–5 min and loaded onto a 10% SDS-polyacrylamide gel. Samples were electrophoresed at 180 V for 40 min. Gels were rapidly transferred to nitrocellulose membranes (Hybond^{TM} ECL^{TM}, Amersham International). Membranes were soaked in blocking buffer (58 mM Na$_2$HPO$_4$, 17 mM NaH$_2$PO$_4$, 6 mM NaCl, 0.2% I-Block (Tropix, Bedford, MA), 0.1% Tween 20, pH 7.4) overnight at 4 °C on an oscillating platform, then incubated 3 h at 25 °C with the primary antibody (mouse anti-PGHS-1 (Oxford Biochemical Research, Oxford, MI) dilution 1:2500; monoclonal anti-PGHS-2 (Transduction Laboratories, Lexington, KY), dilution 1:2500). Membranes were washed in buffer (58 mM Na$_2$HPO$_4$, 17 mM NaH$_2$PO$_4$, 68 mM NaCl, 0.1% Tween 20, pH 7.4), then incubated with the secondary antibody (goat anti-mouse IgG-conjugated alkaline phosphatase or goat anti-rabbit IgG-conjugated alkaline phosphatase, 1:10,000, Tropix) for 30 min at 25 °C in a rotary oven. For detection, membranes were equilibrated in assay buffer (0.1 M diethanolamine, 1 mM MgCl$_2$, pH 10) twice for 2 min each time, incubated in 5 ml of assay buffer containing 0.25 mM CSPD (Western-light^{TM}, Tropix) and 1:20 Nitro-Block (Western-light^{TM}, Tropix) for 1 min, then rapidly exposed to x-ray film (Hyperfilm-ECL, Amersham International) or a PhosphorImager plate.

RESULTS

KA- and ECS-induced Seizures Elevate PGS-2 and zif-268 mRNA Levels in Brain—The abundance in the brain of several immediate-early gene transcripts shows a rapid and transient increase in response to electrically and chemically induced seizures (22, 23). We had previously shown that a single ECS induced *zif*-268 mRNA expression in the rat hippocampus and cerebral cortex, and this induction was sensitive to the intracellular PAF receptor antagonist BN 50730 (9). We were, therefore, interested in determining if PGS-2 is regulated by a similar mechanism in the ECS model, and if both are regulated in a similar manner in the KA-induced seizure model.

KA-induced seizures elevated mRNA levels of both PGS-2 and *zif*-268. However, there were differences in the magnitude and duration of the responses, and differences in the levels of response of the various neuroanatomical regions (Fig. 1). Induction of PGS-2 mRNA 2 h after treatment was highest in the hippocampus (35-fold), followed by the cerebral cortex (8-fold). Small changes were observed in the brain stem and striatum, whereas there was no change in cerebellum. Similar to PGS-2, the largest induction of *zif*-268 mRNA was in hippocampus (5.5-fold), followed by cerebral cortex (4.8-fold). Again, smaller changes were found in brain stem and striatum and no change in cerebellum.

Hippocampal PGS-2, but Not zif-268 mRNA Accumulation Is Much Greater in KA- versus ECS-induced Seizures—The time courses of the KA-induced up-regulation of PGS-2 and *zif*-268 mRNA in hippocampus were distinct from each other and different from the time courses of expression in the ECS model. In both models, the peak of PGS-2 mRNA induction was later than *zif*-268. In KA-treated animals, PGS-2 reached a 71-fold increase over controls in 3 h, whereas *zif*-268 peaked at 1 h with a 10-fold increase. PGS-2 mRNA levels increased 5.2-fold at 12 h (Fig. 2*A*). At 24 h, the PGS-2 mRNA levels were still above baseline (data not shown). With the ECS model, PGS-2

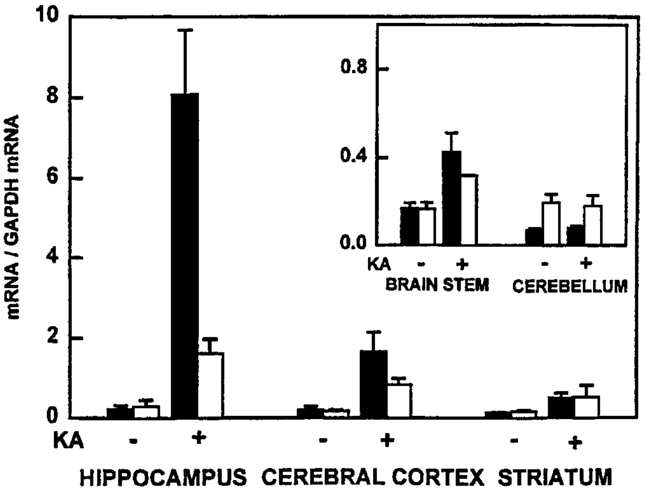

FIG. 1. **Neuroanatomical distribution of PGS-2 mRNA (*closed bars*) and *zif*-268 mRNA (*open bars*) up-regulation in rat brain after KA administration.** Experimental animals (+) received a single ip injection of KA, whereas controls (−) received saline. RNA was isolated 2 h after injection, and PGS-2, *zif*-268, and GAPDH mRNAs were detected by Northern analysis. (n = 6, error bars ± S.D.).

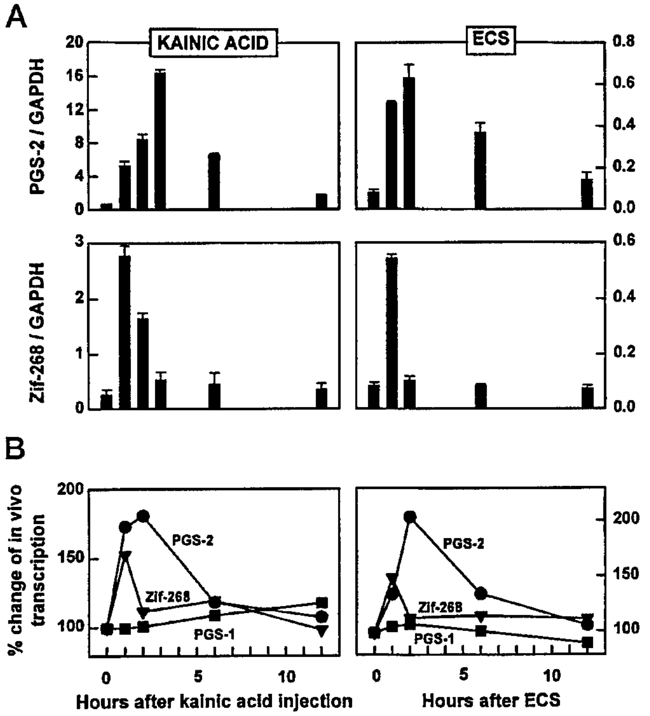

FIG. 2. **Time course of changes in relative mRNA abundance and transcriptional activity in hippocampus after KA treatment or a single ECS.** *A*, relative abundance of PGS-2 and *zif*-268 mRNAs compared to GAPDH mRNA, as assessed by Northern analysis. (n = 9–12 for each time point from 3 separate experiments, error bars ± S.D.). *B*, transcriptional activity of PGS-2, PGS-1, and *zif*-268 genes assessed by nuclear run-on transcription (n = 3–4 from 3 separate experiments). Data are normalized to transcriptional activity of GAPDH.

mRNA increased 10-fold over controls at 2 h, and *zif*-268 increased 8.5-fold after 1 h. Additionally, levels of PGS-2 mRNA returned to basal values by 24 h. The delayed induction of PGS-2 relative to transcription factor immediate-early genes is a well-described phenomenon of many *in vivo* and cell culture systems. The differences between the two seizure models are the most significant observations. When the peak induction of *zif*-268 mRNA levels were similar in magnitude in both models, PGS-2 induction was seven times greater in KA- than in ECS-induced seizures.

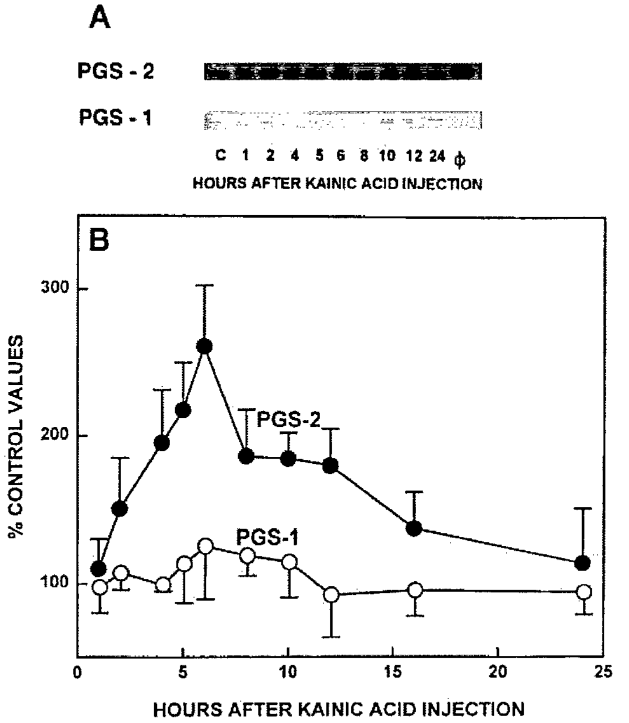

FIG. 3. **Time course of PGS-2 and PGS-1 protein accumulation in rat hippocampus after KA treatment.** *A*, representative Western blots of hippocampal protein extracts using PGS-2 and PGS-1 antibodies. The positive control for PGS-2 (ϕ) is from lipopolysaccharide-induced macrophages. *B*, quantification of Western blots to assess relative induction of PGS-2 and PGS-1 protein in experimental (KA-treated) *versus* control (saline-treated) (n = 8–12 from 3 separate experiments, error bars ± S.D.).

Changes in PGS-2 and zif-268 Expression during Seizures Involve Transcriptional Regulation—The changes in mRNA abundances during KA- and ECS-induced seizures may be due to a number of seizure-induced changes in hippocampus, including enhanced transcriptional activity, increased availability of factors that prolong mRNA stability, and/or changes in translational modulation. To determine if transcriptional activation is a component of PGS-2 and *zif*-268 induction in the seizure models, nuclear run-on transcription assays were performed. The profiles of PGS-2 transcriptional activation qualitatively match the changes found in mRNA abundance (Fig. 2*B*), suggesting that transcriptional activation is a major factor in PGS-2 induction. The relative induction of PGS-2 transcriptional activation was, however, less than the relative increase in mRNA levels. The methodologies used to obtain these two sets of values are very different and may not be readily comparable. Therefore, we can only conclude that the *in vitro* transcription profile is analogous to that of relative mRNA abundance. Similar comparative patterns can be observed between *in vitro* transcription and mRNA abundance for *zif*-268.

We also monitored transcriptional activation of PGS-1, the constitutively expressed isoform of PGS. As expected, there was no significant induction up to 6 h; however, there was a small increase in KA-injected animals after 12 h. ECS did not trigger any activation.

Induction of Hippocampal PGS-2 mRNA Expression during KA-induced Seizures Is Reflected in Levels of Protein Expression—The induction of PGS-2 transcriptional activity and mRNA accumulation in hippocampus during KA-induced seizures was matched by an increase in protein levels. Western blotting experiments (Fig. 3) showed a peak of PGS-2 protein

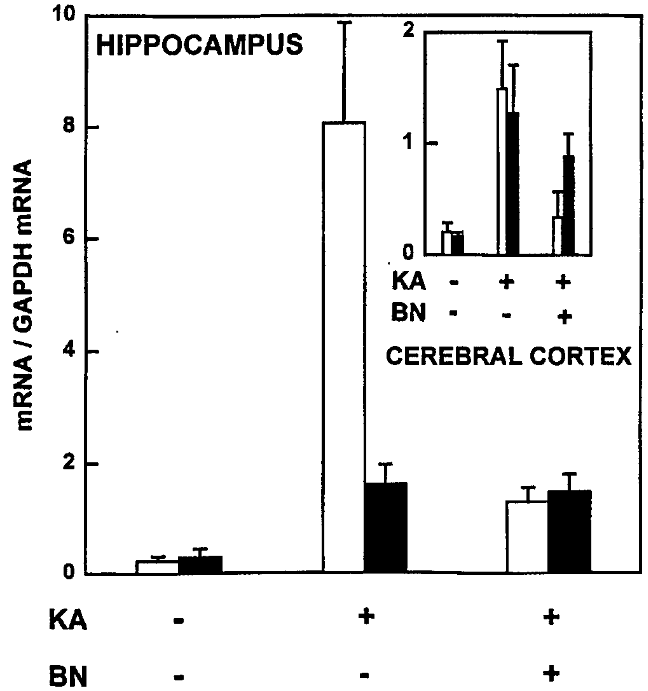

FIG. 4. **Inhibition by BN 50730 of KA-induced PGS-2 mRNA (*open bars*) and *zif*-268 mRNA (*closed bars*) accumulation in rat hippocampus and cerebral cortex.** Animals were pretreated intracerebroventricularly with BN 50730 in Me_2SO (experimental) or Me_2SO alone (control) 15 min before KA injection. RNA was extracted for Northern blot analysis 2 h after KA injection (n = 6 from 2 separate experiments, error bars ± S.D.).

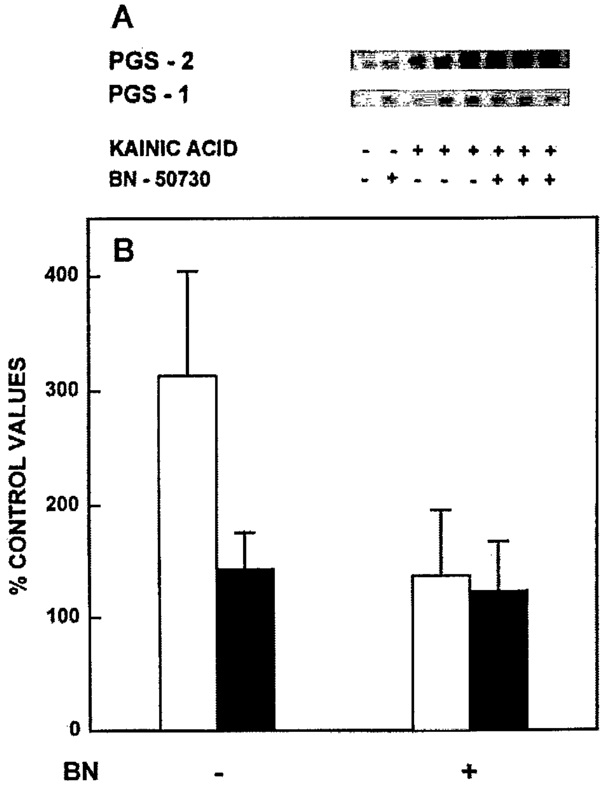

FIG. 5. **Inhibition by BN 50730 pretreatment of KA-induced PGS-2 protein accumulation (*open bars*), but not of endogenous PGS-1 (*closed bars*) in rat hippocampus.** *A*, representative Western blots. BN 50730 or vehicle treatments were as for Fig. 4. Samples were collected 6 h after KA treatment. *B*, quantification of Western blot data expressed as a percent increase over control (vehicle-pretreated) values. (n = 10–12 from 3 separate experiments, error bars ± S.D.).

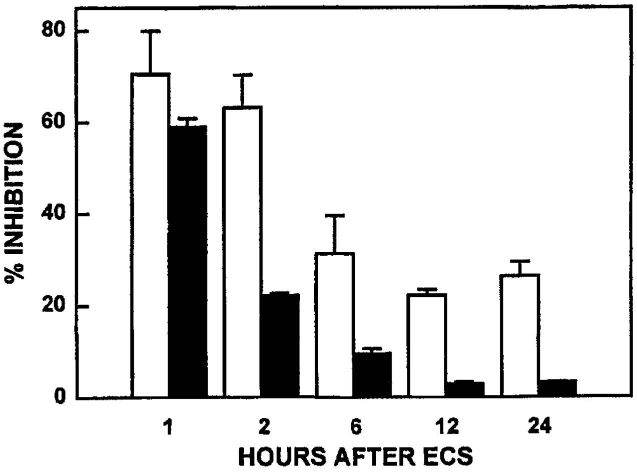

FIG. 6. **Inhibition by BN 50730 pretreatment of ECS-induced PGS-2 mRNA (*open bars*) and *zif*-268 mRNA (*closed bars*) accumulation in rat hippocampus.** The data are expressed as the percentage by which drug treatment decreases the relative mRNA abundance *versus* vehicle treatment. Relative mRNA abundance was measured against GAPDH mRNA. (n = 9 from three separate experiments, error bars ± S.D.).

levels at 6 h. The time course profile showed a shoulder that may represent a smaller, overlapping peak at 12 h. PGS-1 protein levels showed no significant changes over the 24-h time course. There was, however, a small increase in PGS-1 protein levels after KA injection, which mirrored that seen in the run-on transcription experiments. Thus far, the significance of this remains to be explored.

BN 50730 Inhibits Seizure-induced PGS-2 mRNA and Protein Expression in Hippocampus and Cerebral Cortex—The intracellular PAF receptor antagonist BN 50730 was more effective in reducing seizure-induced early gene expression, when administered intracerebroventricularly (icv) as compared with the ip route (9). Therefore, in the present study, BN 50730 was given icv 15 min before KA treatment. Fig. 4 shows that BN 50730 reduced the seizure-induced accumulation of PGS-2 mRNA in hippocampus and cerebral cortex by about 90% at 2 h, but was much less effective against *zif*-268 mRNA accumulation. This was matched by a similar attenuation of PGS-2 protein accumulation in the hippocampus (Fig. 5). Under the same conditions, expression of PGS-1 protein was unaffected. Using the ECS model, when rats were pretreated by icv with BN 50730 15 min before stimulation, PGS-2 and *zif*-268 mRNA accumulation in rat hippocampus was strongly inhibited with the inhibition for both PGS-2 and *zif*-268 peaking 1 h after injection (Fig. 6). The decreased effectiveness of the drug after this time presumably represents a combination of diffusion or transport away from the site of action and/or metabolic inactivation.

The dose of KA used in the experiments involving pretreatment with BN 50730 was reduced from 10 mg/kg for the other experiments to 6 mg/kg. The higher dose of kainic acid in BN 50730-treated animals produced extremely severe seizures and high mortality. We propose that the solvent for BN 50730 (Me_2SO) increased the permeability of the blood-brain barrier to KA. Only around 1% of KA delivered ip normally reaches the brain (24), and, thus, any increase in blood-brain barrier permeability could easily lead to overdosage.

DISCUSSION

The data presented here implicate PAF-stimulated signal transduction pathways as major components of the seizure-induced expression of PGS-2 in brain. We also confirm previous studies demonstrating seizure-induced increases in PGS-2 mRNA levels in brain (17, 25, 26). In addition, we show that accumulation of PGS-2 mRNA correlates with transcriptional activation of the gene and accumulation of PGS-2 protein. Furthermore, when the effects of ECS were compared with KA-induced seizures, we demonstrate that PGS-2, but not the zinc-finger transcription factor *zif*-268, is subject to additional hippocampal up-regulation in the more severe seizure model.

The inhibitory effect of BN 50730 on seizure-induced PGS-2 and *zif*-268 implies that they share a common requirement for stimulation of the intracellular form of the PAF receptor in the mechanisms by which both genes are induced under these conditions. BN 50730 is a competitive antagonist of PAF, binding to an intracellular site, but not to synaptic membranes (9). Nonetheless, the different kinetics of induction and the selective increase in response of hippocampal PGS-2 induction in KA- *versus* ECS-induced seizures suggest that other components of the induction mechanisms are distinct. BN 50730 almost totally abolishes KA-induced PGS-2 mRNA and protein accumulation. Therefore, it is likely that PGS-2 expression responds either to increased levels of PAF and/or with an increased sensitivity of the PAF receptor. PAF induces mouse PGS-2 promoter-driven luciferase activity transfected in neuroblastoma cells (NG 108–15 or SH-SY5Y) and in NIH 3T3 cells, and BN 50730 inhibits this effect (15). Increased PAF content was detected in brain during seizures (4); however, it is difficult to assess the actual increases of PAF that might occur intracellularly in the brain due to the presence of a wide variety of PAF acetylhydrolases (27, 28). *zif*-268 expression is less sensitive than PGS-2 expression to BN 50730 inhibition in the ECS model, does not show an augmented response in hippocampus in the KA model, and, in the latter model, shows little response to BN 50730. This suggests that either *zif*-268 is induced by different mechanisms in the two seizure models or that ECS is sufficient to maximally stimulate expression. The induction of PGS-2 in hippocampus by KA is elevated and prolonged in neuroanatomical regions known to exhibit selective neuronal apoptosis in response to this agonist (29).

PGS-2 expression in the normal rat brain is localized to the post-synaptic structures of discrete groups of neurons in the forebrain and is enriched in the hippocampus and cerebral cortex (18, 30). The onset of expression in the developing rat correlates with the early postnatal period when synaptic remodeling is most active. Even when expression is up-regulated by ECS, the mRNA is still localized to neurons and is not detected in glial or vascular cells (17). Hence, the up-regulation of PGS-2 expression in response to KA is probably due to additional stimulation of the neuronal pathway by the multiple KA-induced seizures. This would suggest an atypical role for the prostaglandins generated by PGS-2 activity. PGS-2 activation is generally thought to be associated with inflammatory events, whereas the constitutive PGS-1 isoform is thought to be responsible for physiological production of prostanoids. It should be added, however, that this strict definition of the roles of the two isoforms is being reassessed (31), particularly in the light of the phenotypes exhibited by knockout mice for the two PGS isoforms (32–34). PGS-1 knockout mice have few phenotypic abnormalities, whereas PGS-2 knockouts have severe renal abnormalities. Thus PGS-2 is required for certain aspects of normal development. Furthermore, testing of various inflammatory models on these animals suggest that both PGS isoforms participate in inflammatory events. At this time, the neurological responses of these mice have not been reported.

The major endogenous inflammatory cells of the brain are glia and microglia. Accordingly, these cell types express relatively large amounts of such components of the inflammatory cascade as secretory and cytosolic PLA_2 and the PAF receptor (35–37). Prostanoids synthesized through PGS-2 activation in neurons are unlikely, therefore, to be directly involved in the inflammatory response. They may, however, be involved in functions related to synaptic transmission and neuronal plasticity. Prostaglandins D_2, E_2, and $F_{2\alpha}$ have been known for some time to have modulatory effects on neurotransmission (5), and, more recently, prostacylin has been found to facilitate excitatory neurotransmission in the hippocampus through a novel receptor subtype (38). Beyond this common property, however, the different prostanoids have diverse and, in some cases, antagonistic effects on the central nervous system. It is important, therefore, to know which species of prostanoids are being synthesized via neuronal PGS-2 both in physiological and pathophysiological states. Temporal changes in the patterns of prostanoids synthesized during seizures using the pilocarpine model of epileptogenesis show production of different prostanoid species at different stages during status epilepticus and the formation of recurrent seizures (39).

The events in which PAF activates PGS-2 expression during seizures sets into motion pathways which can either elicit neuroprotection or lead to neuronal damage. PAF is not stored in cells but is rapidly synthesized in response to stimuli. In addition, it is a short-lived molecule, being rapidly degraded by a specific PAF acetylhydrolase (28). Nonetheless, by inducing PGS-2, this transient signal can have long-term effects on neuronal function. The localization of PGS-2 protein in the nuclear envelope and perinuclear endoplasmic reticulum (40) suggests that, in addition to paracrine effects on the cell-surface prostanoid receptors, the prostanoids produced through PGS-2 activation may have effects at the nucleus. Thus, PAF might be eliciting cascades of gene expression in addition to those potentially initiated by activation of transcription factor genes such as *zif*-268. To fully understand the significance of seizure-induced PGS-2 expression in hippocampus, the types and functions of PGS-2 derived prostanoids synthesized in response to seizures will have to be better defined. With this information, the consequences of BN 50730 inhibition of PGS-2 expression can be more fully assessed.

REFERENCES

1. Bazan, N. G. (1970) *Biochim. Biophys. Acta* **218,** 1–10
2. Birkle, D. L., and Bazan, N. G. (1987) *J. Neurochem.* **48,** 1768–1778
3. Herrting, G., and Seregi, A. (1989) *Ann. N. Y. Acad. Sci.* **559,** 84–99
4. Kumar, R., Harvey, S., Kester, N., Hanahan, D., and Olsen, M. (1988) *Biochim. Biophys. Acta* **963,** 375–383
5. Shimizu, T., and Wolfe, L (1990) *J. Neurochem.* **55,** 1–15
6. Clark, G. D., Happel, L. T., Zorumski, C. F., and Bazan, N. G. *Neuron* **9,** 1211–1216
7. Kato, K., Clark, G. D., Bazan, N. G., and Zorumski, C. F. (1994) *Nature* **367,** 179–182
8. Squinto, S. P., Block, A. L., Braquet, P., and Bazan, N. G. (1989) *J. Neurosci. Res.* **24,** 558–566
9. Marcheselli, V. L., and Bazan, N. G. (1994) *J. Neurosci. Res.* **37,** 54–61
10. Honda, Z., Nakamura, M., Miki, I., Minami, M., Wantabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T., and Shimizu, T. (1991) *Nature* **394,** 342–346
11. Wieraszko, A., Li, G., Kornecki, E., Hogan, M. V., and Ehrlich, Y. H. (1993) *Neuron* **10,** 553–557
12. Jerusalinsky, D., Fin, C., Quillfeldt, J. A., Ferreira, M. B., Schmitz, P. K., Da Silva, R. C., Walz, R., Bazan, N. G., Medina, J. H., and Izquierdo I. (1994) *Behav. Neural. Biol.* **62,** 1–3
13. Izquierdo, I., Fin, C., Schmitz, P. K., Da Silva, R. C., Jerusalinsky, O., Quillfeldt, J. A., Ferreira, M. B., Medina, J. H., and Bazan, N. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 5047–5051
14. Marcheselli, V. L., Rossowska, M., Domingo, M. T., Braquet, P., and Bazan, N. G. (1990) *J. Biol. Chem.* **265,** 9140–9145
15. Bazan, N. G., Fletcher, B. S., Herschman, H. R., and Mukherjee, P. K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5252–5256
16. Herschman, H. R. (1996) *Biochim. Biophys. Acta* **1299,** 125–140
17. Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A., and Worley, P. F. (1993) *Neuron* **11,** 371–386
18. Kaufmann, W. E., Worley, P. F., Pegg, J., Bremer, M., and Isakson, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 2317–2321
19. Marcheselli V. L., and Bazan N. G. (1994) *Soc. Neurosci. Abstr.* **20,** 1053
20. Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
21. Thompson, R. J. (1973) *J. Neurochem.* **21,** 19–40
22. Morgan, J. L., Cohen D. R., Hempstead, J. L., and Curran, T. (1987) *Science* **237,** 192–197

23. Saffen, D. W., Cole, A. J., Worley, P. F., Christy, B. A., Ryder K., and Baraban, J. M. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 7795–7799
24. Sperk, G. (1994) *Prog. Neurobiol.* **42,** 1–32
25. Chen, J., Marsh, T., Zhang, J. S., and Graham, S. H. (1995) *NeuroReport* **6,** 245–248
26. Adams, J., Collaço-Moraes, Y., and de Belleroche, J. (1996) *J. Neurochem.* **66,** 6–13
27. Hattori, M., Arai, H., and Inoue, K. (1993) *J. Biol. Chem.* **268,** 18748–18753
28. Bazan, N. G. (1995) *Nature* **374,** 501–502
29. Pollard, H., Charriaut-Marlangue, C., Cantagrel, S., Represa, A., Robain, O., Moreau, J., and Ben-Ari, Y. (1994) *Neuroscience* **63,** 7–18
30. Breder, C. D., DeWitt, D., and Kraig, R. P. (1995) *J. Comp. Neurol.* **355,** 296–315
31. DeWitt, D., and Smith, W. L. (1995) *Cell* **83,** 345–348
32. Langenbach, R., Morham, S. G., Tiano, H. F., Loftin, C. D., Ghanayem, B. I., Chulada, P. C., Mahler, J. F., Lee, C. A., Goulding, E. H., and Kluckman, K. D. (1995) *Cell* **83,** 483–492
33. Morham, S. G., Langenbach, R., Loftin, C. D., Tiano, H. F., Vouloumanos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., and Lee, C. A. (1995) *Cell* **83,** 473–482
34. Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B.. Contel, N. R., Eng, V. M., Collins, R. J., and Czerniak P. M. (1995) *Nature* **378,** 406–409
35. Sun, G. Y., and Hu, Z. Y. (1995) *Prog. Brain. Res.* **105,** 231–238
36. Stephenson, D. T., Manetta, J. V., White, D. L., Chiou, X. G., Cox, L., Gitter, B., May, P. C., Sharp, J. D., Kramer, R. M., and Clemens, J. A. (1994) *Brain. Res.* **637,** 97–105
37. Mori, M., Aihara, M., Kume, K., Hamanoue, M., Kohsaka, S., and Shimizu, T. (1996) *J. Neurosci.* **16,** 3590–3600
38. Takechi, H., Matsumura, K., Watanabe, Y., Kato, K., Noyori, R., Suzuki, M., and Watanabe, Y. (1996) *J. Biol. Chem.* **271,** 5901–5906
39. Naffah-Mazzacoratti, M. G., Bellissimo, M. I., and Cavalheiro, E. A. (1995) *Neurochem. Int.* **27,** 461–466
40. Reiger, M. K., DeWitt, D. L., Schindler, M. S., and Smith W L. (1993) *Arch. Biochem. Biophys.* **301,** 439–444

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Synergy by Secretory Phospholipase A_2 and Glutamate on Inducing Cell Death and Sustained Arachidonic Acid Metabolic Changes in Primary Cortical Neuronal Cultures*

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Secretory and cytosolic phospholipases A_2 ($sPLA_2$ and $cPLA_2$) may contribute to the release of arachidonic acid and other bioactive lipids, which are modulators of synaptic function. In primary cortical neuron cultures, neurotoxic cell death and [^{3}H]arachidonate metabolism was studied after adding glutamate and $sPLA_2$ from bee venom. $sPLA_2$, at concentrations eliciting low neurotoxicity (≤100 ng/ml), induced a decrease of [^{3}H]arachidonate-phospholipids and preferential reesterification of the fatty acid into triacylglycerols. Free [^{3}H]arachidonic acid accumulated at higher enzyme concentrations, below those exerting highest toxicity. Synergy in neurotoxicity and [^{3}H]arachidonate release was observed when low, nontoxic (10 ng/ml, 0.71 nM), or mildly toxic (25 ng/ml, 1.78 nM) concentrations of $sPLA_2$ were added together with glutamate (80 μM). A similar synergy was observed with the $sPLA_2$ OS2, from Taipan snake venom. The NMDA receptor antagonist MK-801 blocked glutamate effects and partially inhibited $sPLA_2$ OS2 but not $sPLA_2$ from bee venom-induced arachidonic acid release. Thus, the synergy with glutamate and very low concentrations of exogenously added $sPLA_2$ suggests a potential role for this enzyme in the modulation of glutamatergic synaptic function and of excitotoxicity.

Membrane unsaturated fatty acid turnover and the synthesis of bioactive lipids are modulated by phospholipases A_2 (PLA_2),[1] ubiquitous mammalian enzymes that catalyze the hydrolysis of *sn*-2-acyl ester bonds of phospholipids (PLs) (1). Arachidonic acid (AA), eicosanoids, and platelet-activating factor (PAF) are bioactive lipids generated through PLA_2 activation (2). Although some PLA_2 are calcium-independent (3, 4), most found in the brain are characterized by calcium dependence (4, 5). PLA_2 are overstimulated in the brain during seizures and ischemia (6–8) as a consequence of increased calcium influx and/or intracellular calcium mobilization, which, in turn, results in the accumulation of bioactive lipids that participate in cell damage (8, 9).

There are secretory and cytosolic PLA_2 ($sPLA_2$ and cPLAs, respectively). $sPLA_2$ (14 kDa) are active at submillimolar concentrations of calcium and do not display selectivity for unsaturated fatty acids at the *sn*-2-position of PLs (4, 5). sPLAs are found in pancreatic secretions (type I), platelets, neurons, mast cells, snake venoms, inflammatory exudates (type II), and bee venom (type III) (4, 5, 10). In contrast, $cPLA_2$ (type IV) has a higher molecular mass (85 kDa), is active at submicromolar Ca^{2+} concentrations, and shows selectivity for *sn*-2-arachidonoyl-PLs (5, 11). $cPLA_2$ is activated by translocation to intracellular and nuclear membranes when there is an agonist-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in the brain (12, 13) as well as in other tissues (4, 14).

Among the neural forms of PLA_2 are (*a*) a calcium-sensitive and arachidonoyl-specific 85-kDa $cPLA_2$ (12, 15, 16), highly expressed in astrocytes (17), other cytosolic calcium-dependent forms (12, 16), and calcium-independent forms (3, 18, 19); and (*b*) membrane-bound forms (15), including a very high molecular mass (180-kDa) form from human temporal cortex (20). Secretory PLA_2 are also present in the brain. The expression of $sPLA_2$ type II is stimulated in the rat brain by ischemia/reperfusion (21) and in cultured astrocytes by inflammatory mediators (22). Moreover, $sPLA_2$ type II is stored in synaptic vesicles and released by depolarization or neurotransmitter stimulation, and its secretion is coupled with the activation of catecholamine release (23). Furthermore, $sPLA_2$ causes activation of Glu release in the rat cerebral cortex (24).

$sPLA_2$ bind to cell surface receptors, the N type and the M type (25–28) identified using $sPLA_2$ purified from snake and bee venoms as ligands. Neurotoxic $sPLA_2$ from Taipan snake venom, OS2, and from bee venom bind to the N-type receptor with high affinity (25, 26). Other $sPLA_2$ such as OS1, also purified from Taipan snake venom, display higher enzymatic activity than the $sPLA_2$ OS2 and bee venom (2.7- and 7-fold higher, respectively) (25). Although OS1 binds with high affinity to M-type receptors (26–28), it does not bind to N-type receptors (25) and is therefore non-neurotoxic.

Activation of $cPLA_2$ mediates the formation of modulators of synaptic transmission such as free AA (8), eicosanoids (29, 30), and PAF (31). Ischemia and seizures promote a rapid increase in brain free AA (6, 7, 32, 33), oxygenated metabolites of AA, and free radicals, all of which are potent neuronal injury mediators (for review, see Ref. 8). A sustained activation of $cPLA_2$ has been reported after ischemia/reperfusion (13, 15). Glu, which causes excitotoxic neuronal damage, increases calcium influx through NMDA receptors in postsynaptic neurons, leading to PLA_2-mediated AA release (34–37), which is blocked by the NMDA antagonist MK-801 (38). Recently, the activation of two calcium-dependent $cPLA_2$ (100 and 14 kDa) by Glu was reported (16).

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[1] The abbreviations used are: PLA_2, phospholipase A_2(s); $sPLA_2$, secretory phospholipase A_2(s); $cPLA_2$, cytosolic phospholipase A_2; PAF, platelet-activating factor; AA, arachidonic acid; PL, phospholipid; CHE, cholesterol ester; TAG, triacylglycerol; FFA, free fatty acids; DAG, diacylglycerol; LDH, lactate dehydrogenase.

Other PLA$_2$ are modulators of membrane PL metabolism and/or generate membrane fusogenic molecules, *i.e.* free fatty acids and lyso-PLs. However, non-AA-specific-sPLA$_2$ contribute, together with the cPLA$_2$, to modulate AA metabolism under physiological conditions and in primed conditions (5, 39–42). sPLA$_2$ venoms have long been known to be neurotoxic (10, 43). For example, sPLA$_2$ from Naja mocambique, as well as the PLA$_2$ activator melittin, have previously been shown to promote neural injury, *in vitro* and *in vivo* (44). The present study has tested the hypothesis that sPLA$_2$ potentiates neurotoxicity by Glu by briefly exposing rat cortical neuronal cultures to this neurotransmitter in the presence of and in the absence of sPLA$_2$. This hypothesis is supported in part by the observation that sPLA$_2$ is released at synapses from vesicles that also store Glu (23). sPLA$_2$ from bee venom and Taipan snake venom OS2, ligands of the N-type sPLA$_2$ receptor, and OS1 from Taipan snake venom, a ligand of the M-type sPLA$_2$ receptor, have been used (25–28). Moreover, sustained changes in neuronal [^{3}H]AA metabolism under these conditions have been observed. Our study has addressed the following: (*a*) the effect of Glu-activated cPLA$_2$ and exogenously added sPLA$_2$ on [^{3}H]AA release from neuronal PLs; (*b*) the action of MK-801 on agonist-induced AA changes and neurotoxicity; (*c*) the neurotoxic potential of exogenously added sPLA$_2$ (OS2 and bee venom sPLA$_2$); and (*d*) the neurotoxic effect of the excitatory neurotransmitter Glu and sPLA$_2$ when added to the cells simultaneously.

EXPERIMENTAL PROCEDURES

Materials—[5,6,8,9,11,12,14,15,-^{3}H]Arachidonic acid ([^{3}H]AA, 185 Ci/mmol) was purchased from DuPont NEN. Silicagel GHL TLC plates were from Analtech, Inc. (Newark, DE). Bovine serum albumin fatty acid-free and lipid standards were from Sigma. Organic solvents (high pressure liquid chromatography grade) were from EM Science. sPLA$_2$ from bee venom and OS1 and OS2 from Taipan snake venom were purified as described previously (25).

Primary Neuronal Cultures—Cortical neuronal cultures were established from 15-day-old rat embryos as described previously (46, 47). Cells were plated in poly-L-lysine-coated dishes at a density of 4 × 10^5 cells/well in 48-well plates for toxicity experiments and 7.5 × 10^5 cells/well in 24-well plates for lipid analysis. The cells were cultured in neuronal culture medium (NCM) containing 10% fetal calf serum and 10% horse serum in a 5% CO$_2$ incubator at 37 °C. Astrocyte proliferation was prevented by adding cytosine arabinoside (10^{-5} M) at day 4 after plating, left on for 3 days, and then replaced with minimum essential medium containing 10% horse serum. Cells were used for experiments at 14–21 days *in vitro*. The percentage of neuronal *versus* glial cells remained at approximately 80 *versus* 20%, respectively, as previously reported (48).

Neuronal Toxicity Assay—Lactate dehydrogenase (LDH) release was used to quantitatively assess cell injury. Cells were treated as described previously (47), and LDH release was measured 20 h after exposure to sPLA$_2$ and/or Glu. Briefly, cells were exposed for 45 min at room temperature to highly purified sPLA$_2$ from bee venom and snake venom (OS1 or OS2) and/or Glu (80 μM) in Locke's solution without magnesium. Locke's solution was exchanged with minimum essential medium without phenol red, and the cultures were returned to the CO$_2$ incubator for 20 h at 37 °C. Using similar cortical neuron culture, this procedure results in minimal LDH release before 12 h and maximal release by 20 h (49). The LDH release was assayed using the Sigma kit with the aid of a DU series 640 Beckman spectrophotometer equipped with a graphic video display.

[^{3}H]Arachidonic Acid Metabolism and Lipid Analysis—Cells were labeled overnight with [^{3}H]AA (0.5 μCi/well, specific activity 184.6 Ci/mmol) in minimum essential medium supplemented with 0.2% fatty acid-free bovine serum albumin. 18–20 h later, the medium of cell cultures was replaced with minimum essential medium. The cells were exposed to Glu and/or sPLA$_2$ as described previously and returned to the CO$_2$ incubator for 2 and 20 h at 37 °C. The supernatants were removed, and 750 μl of methanol was added to each well. The cells were scraped and transferred into a glass tube, and chloroform was added in the following proportion: chloroform:methanol, 2:1 (v/v). Lipids were extracted by sonication for 30 min, and the lipid extracts were washed following the procedure of Folch *et al.* (50). Aliquots in duplicate were taken to determine total [^{3}H]AA incorporation by liquid scintillation counting.

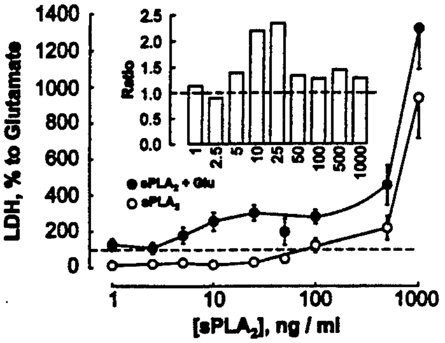

FIG. 1. **Secretory phospholipase A$_2$ from bee venom and Glu elicit synergy of neurotoxicity.** Rat cortical neurons were treated for 45 min at room temperature in Locke's solution with Glu (80 μM) and with increasing concentrations of sPLA$_2$ from 1 ng/ml (71 pM) to 10^3 ng/ml (71 nM) in the presence or absence of Glu (80 μM). The media were assayed for LDH release 20 h later. LDH released by Glu in each plating was considered as 100%, and all other values were normalized to a percentage of the Glu-LDH value. Mean values ± S.D. from at least n = 10 individual determinations are shown. Values of LDH for 25 ng/ml sPLA (130%) or higher were significantly above Glu alone ($p < 0.05$). The *dotted line* indicates a Glu value (100%) attained at 100 ng/ml sPLA$_2$ concentration. *Inset*, plotted values are the ratio between percentage of LDH release induced by sPLA$_2$ added together with Glu divided by the sum of the percentage LDH values obtained with sPLA$_2$ and Glu (100%) assessed individually in the same plating. A ratio of 1 (*dotted line*) indicates that the effects are additive.

Neutral lipids and total PLs were isolated by monodimensional TLC on precoated, 0.25-mm-thick, silica gel GHL plates using hexane:diethyl ether:acetic acid, 40:60:1.3 (v/v/v), as a developing solvent. For the results presented in Table I, the composition of the chromatographic solvent was changed to 60:40:1.3 (v/v/v) in order to isolate cholesterol ester (CHE) from triacylglycerols (TAG) that run together in the first system. The results indicated that changes observed in previous experiments in the CHE plus TAG fraction were due mainly to TAG. Lipid standards were added to the samples as a carrier and were spotted in a parallel line on the TLC plate to individualize each lipid band. TLC plates were developed with iodine, lipid bands were scraped, and the radioactivity was determined in a Beckman scintillation counter.

Statistical Analysis—The significance of the data was evaluated with Student's t test for unpaired data. Statistical values were considered significantly different when $p \leq 0.05$.

RESULTS

Potent Neurotoxicity Elicited by sPLA$_2$—Neurotoxicity of sPLA$_2$ from bee venom, added alone or with Glu (80 μM) to primary neuronal cortical cultures, was studied by measuring LDH release (Fig. 1). A neurotoxic concentration of Glu (47) resulted in 92 ± 7% increase in LDH release compared with control cells (n = 54 from 10 different platings). No significant toxicity was observed with bee venom sPLA$_2$ concentrations up to 10 ng/ml (Fig. 1*A*). At higher concentrations up to 10^3 ng/ml, the neurotoxicity displayed by sPLA$_2$ was biphasic. First, there was a dose-dependent increase in LDH release (up to 500 ng/ml, EC$_{50}$ = 7.1 nM), followed by a sharp 4.3-fold increase in LDH at 10^3 ng/ml (EC$_{50}$ = 57 nM). The sPLA$_2$ neurotoxicity at 100 ng/ml (7.1 nM) was similar to that of 80 μM Glu (Fig. 1). An additive neurotoxic effect was observed when cells were exposed simultaneously to 80 μM Glu and low (1–5 ng/ml) or high (50–10^3 ng/ml) sPLA$_2$ concentrations (Fig. 1, *inset*). The combination of sPLA$_2$ in a concentration range of 10–25 ng/ml with Glu led to a significant synergy on LDH release, resulting in values 2.3–2.4-fold higher than those of the additive effects of sPLA$_2$ and Glu.

The sPLA$_2$ OS2 purified from snake venom was found to be more potent as a neurotoxin than the sPLA$_2$ from bee venom. For example, at 25 ng/ml OS2 was approximately 2.7-fold more

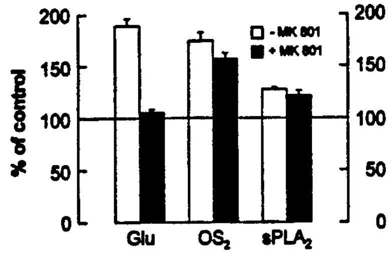

FIG. 2. **MK-801 elicits neuroprotection against Glu but not against $sPLA_2$ from bee venom or OS2.** Rat cortical neurons were treated as described in the legend to Fig. 1 and assayed for LDH release. Values are normalized to percentage of control wells, treated with Locke's solution only; wells treated with MK-801 received MK-801 for 10 min prior to the addition of toxins or Glu and remained in the wells during treatment. Mean values are shown ± S.E. from a representative set of culture plates derived from the same plating, treated and assayed on the same day. The *dotted line* (100%) indicates control, (nontoxic) levels for LDH. MK-801 blocks Glu ($p < 0.001$) by 100% but does not affect OS2 ($p < 0.11$) and bee venom $sPLA_2$ ($p < 0.33$) neurotoxicity.

toxic than bee venom $sPLA_2$ at the same concentration (Fig. 2). Furthermore, under conditions where the noncompetitive NMDA antagonist MK-801 blocked 100% of 80 μM Glu toxicity, MK-801 partially blocked OS2, but not bee venom $sPLA_2$-induced toxicity. OS1 did not evoke neuronal death even at 10 μg/ml (LDH percentage above control = 18 ± 9%).

$sPLA_2$ Promotes Arachidonic Acid Release from Phospholipids—[^{3}H]AA-prelabeled neuronal cells were exposed to different concentrations of bee venom $sPLA_2$ for 45 min and further incubated for 20 h (Fig. 3). No differences were observed in total [^{3}H]AA labeling recovered per dish at very low, nontoxic $sPLA_2$ concentration (1 ng/ml). At higher concentrations (25–50 ng/ml), the recovery was decreased by 10% and by 20–30% at more toxic concentrations (500–10^3 ng/ml), reflecting cell loss and matching the neurotoxicity assays (Fig. 1). After 20 h the [^{3}H]AA distribution displayed a concentration-dependent loss of [^{3}H]AA-PLs paralleled by an increase in free [^{3}H]AA, [^{3}H]AA-TAG and [^{3}H]AA-DAG. A significant loss in PL labeling was observed even at the lowest $sPLA_2$ concentration (−7%, $p < 0.05$), reaching values 50% lower at the highest toxic concentrations (500–10^3 ng/ml). Up to 100 ng/ml $sPLA_2$, the loss of [^{3}H]AA from phospholipids (−29%) was paralleled by its active reesterification into TAG, which showed a 25% increase above the control value. Within this range of $sPLA_2$ concentration, free [^{3}H]AA showed a small yet significant gradual increase, reaching values 2- and 4.5-fold higher than controls at 1 ng/ml and 100 ng/ml, respectively. The [^{3}H]AA-TAG labeling plateaued at 500 ng/ml $sPLA_2$. This was paralleled by a large increase in free [^{3}H]AA accumulation, which reached a value 20-fold higher than control. [^{3}H]DAG labeling was very low, displaying the same pattern of changes as free [^{3}H]AA and reaching a 2-fold increase in percentage of labeling at high $sPLA_2$ concentration (500 ng/ml).

Triacylglycerols Are a Finite Reservoir for the Uptake of [^{3}H]AA Released by $sPLA_2$ and Glu—To ascertain if [^{3}H]AA released by bee venom $sPLA_2$ was acylated into TAG and whether or not this correlated with neurotoxicity, the following experiment was performed. The [^{3}H]AA metabolism as affected by a nontoxic concentration of $sPLA_2$ (1 ng/ml) and by a toxic concentration of Glu (80 μM), added individually or combined, was studied at 2 and 20 h after treatment with the agonists (Fig. 4). $sPLA_2$ induced a similar decrease in [^{3}H]AA-PL labeling both at 2 and 20 h. Differences were observed, however, in the distribution of labeling between free [^{3}H]AA and [^{3}H]AA-TAG. Free [^{3}H]AA accumulation was greater at 2 h, decreasing by 20 h concomitantly with an increase in [^{3}H]AA-TAG.

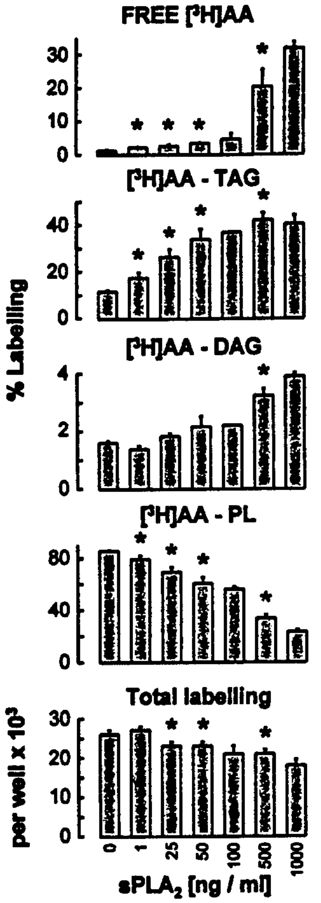

FIG. 3. **Hydrolysis of [^{3}H]arachidonoyl-phospholipids from cortical neurons by $sPLA_2$ from bee venom.** Cortical neuronal cells cultures, labeled overnight with 0.5 μCi/well [^{3}H]AA, were treated for 45 min with increasing concentrations of $sPLA_2$. The percentage of labeling of neuronal free [^{3}H]AA, [^{3}H]AA-TAG, [^{3}H]AA-DAG, and [^{3}H]AA-PLs and total labeling recovered per dish were assessed 20 h later. Mean values ± S.E. from values obtained with 10 different platings are shown. Mean values ± dispersion from the mean are shown for $sPLA_2$ concentrations of 100 ng/ml and 10^3 ng/ml ($n = 2$). An *asterisk* denotes statistic significantly different from control ($p < 0.05$).

Glu alone triggered a similar loss in [^{3}H]AA-PL compared with $sPLA_2$ by 2 h; however, by 20 h, loss of [^{3}H]AA from PL was 2.8-fold greater than at 2 h. After treatment with Glu alone, free [^{3}H]AA and [^{3}H]AA-TAG varied as a function of time (similar to when $sPLA_2$ was added alone), with higher accumulation of free [^{3}H]AA by 2 h and a preferential reesterification of [^{3}H]AA into TAG by 20 h.

$sPLA_2$ and Glu added together greatly magnified the pattern of [^{3}H]AA changes as a function of time. A synergy on free [^{3}H]AA accumulation was observed due to an apparently less efficient esterification into TAG. By 20 h the level of free [^{3}H]AA reached 1.8–2-fold higher values than when both agonists were individually added. The loss of [^{3}H]AA from PLs was additive, as was the accumulation of [^{3}H]AA-DAG induced at 2 and 20 h.

MK-801 Does Not Block Arachidonic Acid Release Induced by $sPLA_2$ from Bee Venom but Partially Blocks the Effect of OS2 from Snake Venom—The involvement of NMDA receptors on AA release from PLs induced by $sPLA_2$ and Glu was investigated by preincubating cells with 300 nM MK-801 for 10 min prior to adding the agonists, followed by lipid analysis 20 h later. Both at low, nontoxic (1 ng/ml) (data not shown) and at higher (25 ng/ml) bee venom $sPLA_2$ concentrations (Table I), MK-801 did not block the release of [^{3}H]AA from PLs. The phospholipid labeling was decreased by 17% ($p < 0.002$), from 87% in controls to 70% in $sPLA_2$-treated cells. Most of the

[^{3}H]AA released from PL (+11%) was found reesterified into TAG (5 *versus* 16% for control and $sPLA_2$-treated, respectively) and to a lesser extent in CHE (+4%, $p < 0.03$), while free [^{3}H]AA labeling was doubled (from 1 to 2%, $p < 0.03$). MK-801 pretreatment did not alter the profile of lipid labeling, *i.e.* the decrease in PLs and the parallel increase in TAG and free AA labeling.

Glu (80 μM), although more toxic than 25 ng/ml bee venom $sPLA_2$ ($sPLA_2$ toxicity 29% compared with Glu; Fig. 1), induced only a 6% ($p < 0.002$) decrease in PL labeling concomitantly with increased labeling of TAG (+2%, $p < 0.004$), CHE (+2%, $p < 0.02$), and FFA (+0.4%, $p < 0.03$). MK-801 pretreatment blocked by 100% Glu-induced PL degradation and other lipid changes. Higher degradation of PLs was observed when bee venom $sPLA_2$ and Glu were added together to the cells (−30%). Labeling of TAG increased by 24%, and labeling of free [^{3}H]AA increased by 3% ($p < 0.03$). MK-801 pretreatment blocked partially the changes induced by bee venom $sPLA_2$ and Glu, leading to the same profile of lipid labeling induced by $sPLA_2$ alone.

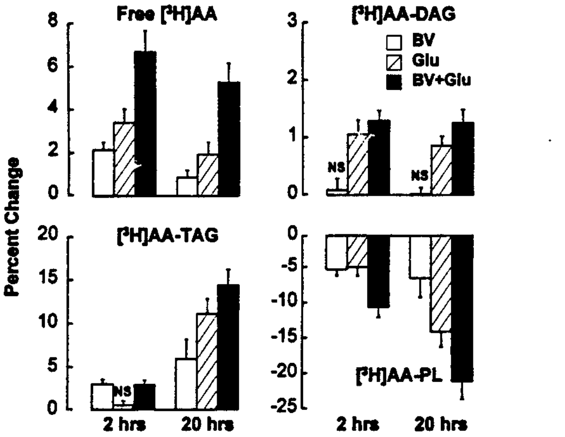

FIG. 4. **$sPLA_2$ and Glu trigger both acute and sustained effects on arachidonic acid metabolism in cortical neurons.** Cortical neuronal cultures prelabeled overnight with [^{3}H]AA (0.5 μCi/well) were treated individually for 45 min with a nontoxic concentration of bee venom $sPLA_2$ (1 ng/ml), Glu (80 μM), or both agonists together. Cells were harvested 2 and 20 h later and the percentage distribution of lipid labeling was determined. *Percent change* represents the percentage of labeling in stimulated cells minus the percentage of labeling of individual lipids in controls. Mean values ± S.E. from at least $n = 5$ determinations for 2 h and $n = 11$ for 20 h are shown. All *percent change* values are significantly different from controls ($p < 0.05$) except for those shown as *NS* (not significantly different).

The $sPLA_2$ from snake venom, OS2, added to the cells at the same concentration as $sPLA_2$ from bee venom (25 ng/ml), induced a much greater degradation of [^{3}H]AA-PLs. MK-801, in contrast to the results with bee venom, partially blocked [^{3}H]AA-PL hydrolysis induced by OS2 when added alone or together with Glu (Table I). Moreover, the total labeling recovered per well treated with OS2 and OS2 plus Glu was decreased by 35%, indicating a massive loss of cells. The DPM/well obtained when the cells were pretreated with MK-801 was similar to controls. Minimal changes in [^{3}H]AA-lipid labeling were observed when the cells were treated with the $sPLA_2$ (25 ng/ml) from snake venom OS1 (data not shown), which does not bind to neuronal membranes and which was found to be non-neurotoxic (see above).

$sPLA_2$ Display a Synergy with Glu in [^{3}H]AA Release from Phospholipids—$sPLA_2$ (25 ng/ml) from snake and bee venoms added with Glu displayed synergy leading to a higher [^{3}H]AA-PL degradation than the sum of the effect of the individual agonists (Table I, Fig. 5). Although the toxicity and PL hydrolysis induced by OS2 was much greater than that of bee venom $sPLA_2$ (Table I), the synergy with Glu was similar, reaching values for PL hydrolysis 1.4-fold higher for both $sPLA_2$ (Fig. 5*C*). A synergy was also observed in the accumulation of [^{3}H]AA-TAG that increased by 2-fold for bee venom $sPLA_2$ and 1.4-fold for OS2 (Fig. 5*B*). The synergy in free [^{3}H]AA accumulation was much greater with OS2 (3.5-fold) than with $sPLA_2$ from bee venom (2-fold) (Fig. 5*A*), and the synergy of $sPLA_2$ plus Glu was blocked by MK-801 (Table I).

Accumulation of Free [^{3}H]AA in Cortical Neurons Precedes the Toxicity Induced by High Concentrations of Bee Venom $sPLA^2$—Treatment of neuronal cultures with increasing concentrations of $sPLA_2$ resulted in increased neurotoxicity (Fig. 1) and higher degradation of AA-PLs (Fig. 4). Changes in lipid labeling plotted as a function of $sPLA_2$ toxicity are shown in Fig. 6. The accumulation of free [^{3}H]AA was minimal and proportional to increased LDH up to 100%, when $sPLA_2$ toxicity was equal to that of 80 μM Glu (≤100 ng/ml $sPLA_2$). Within this range of neurotoxicity, most of the [^{3}H]AA released from PLs (−30%) was reesterified into TAG. While PLs displayed a gradual loss of [^{3}H]AA up to LDH values of 200% (−50% decrease in PL labeling), accumulation of free [^{3}H]AA peaked between LDH values of 100 and 200%. This increase in free [^{3}H]AA preceded a 4.3-fold increase in LDH release observed for $sPLA_2$ concentrations between 500 ng/ml (217%) and 10^3 ng/ml (937% LDH).

TABLE I
Percentage of labeling of [^{3}H]AA-lipids from neuronal cells in culture 20 h after treatment with $sPLA_2$ and glutamate

Cells were labeled overnight with [^{3}H]AA and then exposed for 45 min to $sPLA_2$ from bee venom (25 ng/ml), OS2 from Taipan snake venom (25 ng/ml), and/or glutamate (80 μM). Vales represent percentage distribution of labeling among neutral lipids and phospholipids recovered from cells. Mean values ± S.E. are shown for the number of individual determinations (n) indicated. For samples with $n = 2$, mean values ± S.D. are shown. Asterisks denote values statistically significantly different from control (Student's t test, $p < 0.05$).

Condition		Labeling					Total
		CHE	TAG	FFA	DAG	PL	
		%	%	%	%	%	*dpm/well*
Control	$n = 8$	5.8 ± 0.8	4.9 ± 0.5	1.0 ± 0.1	1.7 ± 0.1	86.6 ± 1.3	261,084 ± 10,723
Glu	$n = 4$	8.2 ± 0.6*	7.3 ± 0.5*	1.3 ± 0.1*	2.3 ± 0.2*	80.8 ± 1.0*	242,010 ± 15,687
Glu + MK-801	$n = 4$	6.7 ± 0.6	4.7 ± 0.1	1.0 ± 0.0	1.5 ± 0.2	86.1 ± 0.6	261,186 ± 24,050
$sPLA_2$	$n = 4$	9.9 ± 1.2*	15.7 ± 1.3*	2.3 ± 0.1*	2.1 ± 0.3	70.1 ± 2.3*	226,002 ± 11,425*
$sPLA_2$ + MK-801	$n = 4$	7.5 ± 1.5	14.6 ± 2.2*	2.3 ± 0.3*	1.8 ± 0.2	73.8 ± 3.2*	251,838 ± 14,374
$sPLA_2$ + GLU	$n = 4$	7.4 ± 1.1	28.8 ± 4.0*	4.0 ± 0.8*	2.9 ± 0.1*	56.9 ± 4.9*	225,108 ± 15,953
$sPLA_2$ + GLU + MK-801	$n = 4$	7.3 ± 0.7	13.5 ± 1.4*	2.2 ± 0.8*	2.5 ± 0.4	74.4 ± 1.2*	229,122 ± 11,353
OS2	$n = 3$	11.3 ± 1.9*	22.2 ± 3.7*	4.7 ± 0.9*	2.1 ± 0.1*	59.6 ± 6.3*	168,961 ± 50,967*
OS2 + MK-801	$n = 2$	6.2 ± 2.0	16.6 ± 4.1	2.3 ± 0.9	2.3 ± 0.1	72.6 ± 6.9	224,448 ± 42,155
OS2 + GLU	$n = 3$	6.0 ± 0.3	32.8 ± 2.6*	15.4 ± 3.3*	3.8 ± 0.2*	41.8 ± 5.8*	173,664 ± 1144*
OS2 + GLU + MK-801	$n = 2$	5.0 ± 0.5	14.8 ± 3.0	2.5 ± 0.6	2.1 ± 0.0	75.5 ± 4.1	233,448 ± 13,950

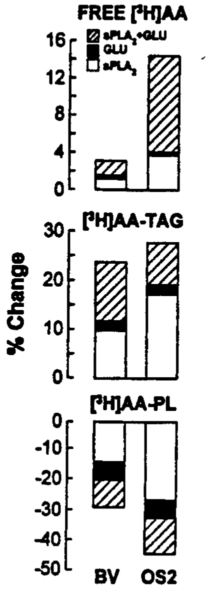

FIG. 5. **Synergistic effect of bee venom $sPLA_2$ plus glutamate on [^{3}H]AA release from phospholipids.** Percentage changes for $sPLA_2$ from bee venom (*BV*) and OS2 from Taipan snake venom and glutamate were calculated from values shown in Table I as described in the legend to Fig. 4. The *open areas* on the *bar graphs* show the changes induced by $sPLA_2$ alone, and the *shaded areas* show changes induced by Glu alone. The additive effects of $sPLA_2$ plus Glu are indicated by the height of *open plus shaded area*; the synergistic effect of $sPLA_2$ and Glu added together is denoted by the *hatched area*, which is *above* and *beyond* the additive areas.

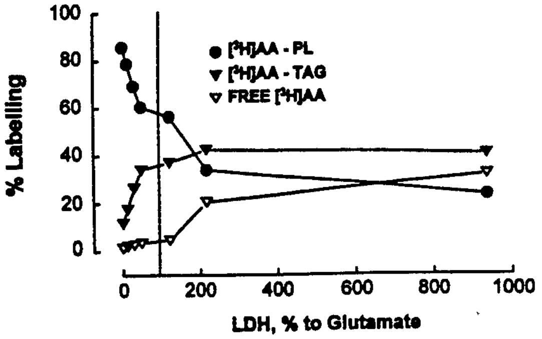

FIG. 6. **$sPLA_2$-induced neurotoxicity correlates with changes in arachidonic acid metabolism.** Plotted values were taken from those shown in Fig. 1 (percentage of LDH release) and Fig. 3 (percentage of lipid labeling) for increasing bee venom $sPLA_2$ concentrations. Cells were treated and data were analyzed as described under "Experimental Procedures." The *vertical dotted line* indicates the percentage of labeling that occurred for $sPLA_2$ concentration (100 ng/ml) with equivalent toxic effect as 80 μM Glu.

DISCUSSION

This study shows that treatment of primary cortical neurons in culture with $sPLA_2$ induces (*a*) a concentration-dependent increase in neurotoxicity; (*b*) sustained activation of [^{3}H]AA mobilization reflected in a gradual loss of [^{3}H]AA from PLs and in an accumulation of free[^{3}H]AA followed by its reesterification into TAG; and (*b*) synergy with Glu (80 μM) for both neurotoxicity and [^{3}H]AA-PL hydrolysis.

Neurotoxicity and sustained changes in AA metabolism, triggered by 45-min exposure of primary cortical neurons to Glu were blocked by the NMDA receptor antagonist MK-801 (Fig. 2, Table I) in agreement with previous studies (34–37, 51–53). Moreover, the release of [^{3}H]AA from PLs was observed 2 h after the treatment of neuronal cultures with Glu, and even greater release was observed 20 h later (Fig. 4). Long lasting changes in AA metabolism may be the result of calcium and protein kinase C-mediated, sustained activation of neuronal $cPLA_2$ by Glu (16). Moreover, increased $cPLA_2$ activity correlates with Glu neurotoxicity and precedes irreversible neuronal injury (16). It is also possible that, as in mast cells (54), Glu may regulate $cPLA_2$ activity at early time points by protein kinase C-mitogen activated protein kinase phosphorylation and later by enhanced expression of the enzyme. Modulation of gene expression and increased protein synthesis are involved in long term cellular responses as in neuronal plasticity or delayed neuronal death. In fact, $cPLA_2$ activation by NMDA-glutamatergic synaptic activity may lead to the formation of PAF, a potent bioactive lipid, which, in turn, mediates the induction of early response genes and subsequent gene cascades (2, 55–57). PAF could also potentiate excitotoxicity by enhancing Glu release (58, 59).

Although the toxicity of Glu (80 μM) was similar to that induced by bee venom $sPLA_2$ (100 ng/ml; Fig. 1), the hydrolysis of [^{3}H]AA-PLs 20 h after Glu treatment (−15%; Fig. 4) was half of that generated by 100 ng/ml bee venom $sPLA_2$ (−29%; Fig. 3). These results and the fact that MK-801 blocked Glu neurotoxicity support the notion that mechanisms other than $cPLA_2$ activation mediated by Glu-activated NMDA-gated calcium channels contribute to its neurotoxic action (8). Glu may also activate metabotropic receptors that, in turn, activate phospholipase C with the release of AA-DAG, a potent activator of protein kinase C (60). Sequential degradation of AA-DAG by diacylglycerol lipases and monoacylglycerol lipases contribute also to increased free [^{3}H]AA (61).

Bee venom $sPLA_2$-dependent sustained changes in [^{3}H]AA-lipid metabolism (2 and 20 h after adding the enzyme) reveal an active release of [^{3}H]AA from PLs, transient accumulation of free [^{3}H]AA, and reesterification into TAG. A similar effect was observed with Glu, with increased labeling of free [^{3}H]AA by 2 h decreasing by 20 h concomitantly with increased [^{3}H]AA-TAG labeling. Interestingly, free [^{3}H]AA was shunted into TAG even when cells were exposed to very low, nontoxic concentrations of $sPLA_2$ (1–10 ng/ml). Thus, the pathway activated by $sPLA_2$ may be physiologically relevant, withholding AA from its conversion to eicosanoids and from exerting effects of its own. AA is a modulator of synaptic function and potentiates Glu-NMDA neurotransmission, leading to excitoxic damage (8). Free AA can be further metabolized to eicosanoids, potent modulators of synaptic function (29, 30), which, when overproduced, become injury mediators (8). TAG may also be a transient reservoir of AA when there is activation of degradative pathways, protecting the cells from the loss of this essential fatty acid. In fact, part of the [^{3}H]AA released during repeated seizures from neuronal membrane PLs in rat brain is shunted into TAG (7). This pathway was also activated in retina by experimental detachment (62), where another polyunsaturated fatty acid, docosahexaenoate (22:6n-3), is actively esterified into TAG. A reversible accumulation of AA-TAG occurs in non-neural cells cultured in the presence of high concentrations of FFA (63, 64). In the present study, even 20 h after transient cell stimulation with nontoxic concentrations of $sPLA_2$, [^{3}H]AA released from PLs remained as [^{3}H]AA-TAG. This indicates long lasting metabolic changes, since between 2 and 20 h post-treatment, PLs did not recover basal labeling, and free [^{3}H]AA was shunted into TAG.

The TAG reservoir appears to have a limited capacity to store AA. The maximum was reached at bee venom $sPLA_2$ concentrations between 50 and 100 ng/ml. AA-PL hydrolysis in neuronal cortical cells was much more sensitive to $sPLA_2$ than toxicity, within a range of LDH release similar to that exerted by Glu (Fig. 6). Thus, for $sPLA_2$ concentrations ≤100 ng/ml (toxicity ≤100% to Glu), the bulk of [^{3}H]AA mobilized from PLs was recovered in TAG. Only when TAG reached a 30% increase in labeling above basal level did further degradation of [^{3}H]AA-PLs induced by higher, more toxic $sPLA_2$ concentrations result in preferential accumulation of free [^{3}H]AA. Taken together these results suggest that as long as the cells are able to shunt AA to TAG, they are protected from accumulation of free AA, and the neurotoxicity of $sPLA_2$ is minimized. Moreover, similar mechanisms allow significant mobilization of [^{3}H]AA from PLs without neurotoxic consequences (*i.e.* at 1 ng/ml $sPLA_2$). This supports the potential physiological relevance of $sPLA_2$ actions in promoting the formation of second messenger modulators of synaptic activity.

Neurotoxicity generated by $sPLA_2$ was biphasic with a linear increase up to 500 ng/ml and a sharp increase thereafter (Fig. 1). The estimated EC_{50} for the two components (7.1 nM and 56.8 nM, respectively) is consistent with the two high affinity binding sites for OS2 in synaptic membranes (25). Moreover, $sPLA_2$ from bee venom competes with OS2 for both binding sites (25). At present there is no information regarding the location of these receptors in the same or different cells or pre- and/or post-synaptic level.

Bee venom $sPLA_2$ at nontoxic (10 ng/ml) and mildly toxic (25 ng/ml) concentrations, when added together with Glu, displayed synergy in neurotoxicity (Fig. 1). Moreover, a 2.3-fold higher toxicity induced by $sPLA_2$ (25 ng/ml) plus Glu was also paralleled by synergy on PL degradation and 2-fold higher accumulation of free [^{3}H]AA. OS2, which is more toxic than $sPLA_2$ from bee venom (Ref. 25 and present results), displayed a more prominent synergy (3.5-fold) on free [^{3}H]AA accumulation. The results reported here open up several questions for future exploration; *e.g.* is Glu-induced $cPLA_2$ activation potentiating $sPLA_2$-mediated degradation of [^{3}H]AA-PLs and cellular toxicity, or *vice versa*? Recent studies carried out in $P388D_1$ macrophages revealed that PAF-induced AA mobilization involves two different PLA_2s (39) and that activation of $cPLA_2$ favors subsequent $sPLA_2$-induced AA release (40). Also, nerve growth factor, a regulator of mast cell function, has been reported to potentiate $sPLA_2$-induced histamine release (65). These observations suggest that $sPLA_2$ actively hydrolyzes lipids in disorganized membrane areas (66). Further studies combining lower, nontoxic concentrations of Glu and mammalian $sPLA_2$ type II, present together with Glu in synaptic vesicles (23), may further elucidate the involvement of both agonists in AA mobilization during glutamatergic synaptic activity.

Up to 25 ng/ml $sPLA_2$ from bee venom induced long lasting changes in AA-PL hydrolysis but did not involve the NMDA-glutamate pathway, since changes in AA metabolism and neurotoxicity were not blocked when $sPLA_2$ stimulation occurred in the presence of MK-801 (Fig. 2, Table I). Moreover, MK-801 partially blocked OS2 effect on AA-PL hydrolysis (Table I). This effect could be related to the origin/structure of the type II $sPLA_2$ from snake venoms and type III $sPLA_2$ from bee venom. Since the toxicity of OS2 at 25 ng/ml is 2.7-fold higher than that of $sPLA_2$ from bee venom at the same concentration (Fig. 2), the possibility of Glu-NMDA involvement at higher bee venom $sPLA_2$ concentrations on [^{3}H]AA-PL hydrolysis cannot be ruled out. Nevertheless, the present results suggest that, at least for OS2, stimulation of Glu release at presynaptic endings followed by its interaction with NMDA receptors may be involved in the acute effects of OS2 resulting in a sustained [^{3}H]AA-PL hydrolysis. Also, it is of interest that the profile of [^{3}H]AA lipid labeling for OS2 stimulation in the presence of MK-801 was identical to that generated by bee venom $sPLA_2$ alone and not blocked by MK-801, indicating a similar receptor-mediated component common for both $sPLA_2$. As previously discussed, this could also be the result of $sPLA_2$ interaction with receptors displaying different affinity for the enzymes.

In summary, this study shows that exogenously added $sPLA_2$ and Glu induce sustained changes in neuronal AA-PL metabolism and that $sPLA_2$ plus Glu exerts synergistic mobilization of AA and subsequent neurotoxicity. The present results, taken together with the recent observation that $sPLA_2$ type II in synaptic vesicles is released together with neurotransmitters (23), open up the possibility that glutamatergic neurotransmission involves the corelease of glutamate and $sPLA_2$. Our observations also imply that excitotoxicity may involve not only glutamate, as currently assumed, but may also involve $sPLA_2$ at the synaptic cleft. Further studies will assess if Glu could potentiate endogenous mammalian $sPLA_2$ actions that could, in turn, stimulate further Glu release. In this connection it is relevant that the synthesis of PAF, a retrograde messenger of long term potentiation (58), may be enhanced by $sPLA_2$ at the synapse. "Cross-talk" between $cPLA_2$ and $sPLA_2$ has recently been suggested in signal transduction events in macrophages (40), and a complex interplay between Glu-activated $cPLA_2$ and $sPLA_2$ could be envisioned at the synapse. Several of these ideas are currently under investigation in our laboratory.

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REFERENCES

1. Van den Bosch, H. (1980) *Biochim. Biophys. Acta* **604,** 191–246
2. Bazan, N. G. (1994) in *Pharmacology of Cerebral Ischemia* (Krieglstein, J., and Oberpichler-Swenk, H., eds) pp. 3–15, Wissenschaftlich Verlagsgeselschaft mbH, Stuttgart
3. Pierik, A. J., Nijssen, J. G., Aarsman, A. J., and Van den Bosch, H. (1988) *Biochim. Biophys. Acta* **962,** 345–353
4. Dennis, E. A. (1994) *J. Biol. Chem.* **269,** 13057–13060
5. Kudo, I., Murakami, M., Hara, S., and Inoue, K. (1993) *Biochim. Biophys. Acta* **117,** 217–231
6. Bazan, N. G. (1970) *Biochim. Biophys. Acta* **218,** 1–10
7. Birkle, D. L., and Bazan, N. G. (1987) *J. Neurochem.* **48,** 1768–1778
8. Bazan, N. G., Rodriguez de Turco, E. B., and Allan, G. (1995) *J. Neurotrauma* **12,** 791–814
9. Nicotera, P., Bellomo, G., and Orrenius, S. (1992) *Toxicology* **32,** 449–470
10. Davidson, F. F., and Dennis, E. A. (1990) *J. Mol. Evol.* **31,** 228–238
11. Fujimori, Y., Murakami, M., Kim, D. K., Hara, S., Takayama, K., Kudo, I., and Inoue, K. (1992) *J. Biochem.* (*Tokyo*) **111,** 54–60
12. Yoshijara, Y., and Watanabe, Y. (1990) *Biochem. Biophys. Res. Commun.* **170,** 484–490
13. Bonventre, J. V., and Koroshetz, W. J. (1993) *J. Lipid Mediators* **6,** 457–471
14. Clark, J. D., Schievella, A. R., Nalefski, E. A., and Lin, L.-L. (1995) *J. Lipid Mediat. Cell. Signal.* **12,** 83–117
15. Rordorf, G., Uemura, Y., and Bonventre, J. V. (1991) *J. Neurosci.* **11,** 1829–1836
16. Kim, D. K., Rordorf, G., Nemenoff, R. A., Koroshetz, W. J., and Bonventre, J. V. (1995) *Biochem. J.* **310,** 83–90
17. Stephenson, D. T., Manetta, J. V., White, D. L., Chiou, X. G., Gitter, B., May, P. C., Sharp, J. D., Kramer, R. M., and Clemens, J. A. (1994) *Brain Res.* **637,** 97–105
18. Hirashima, Y., Farooqui, A. A., Mills, J. S., and Horrocks, L. A. (1992) *J. Neurochem.* **59,** 708–714
19. Yang, H.-C., Farooqui, A., and Horrocks, L. A. (1994) *Biochem. J.* **299,** 91–95
20. Ross, B. M., Kim, D. K., Bonventre, J. V., and Kish, S. J. (1995) *J. Neurochem.* **64,** 2213–2221
21. Lauritzen, I., Heurteaux, C., and Lazdunski, M. (1994) *Brain Res.* **651,** 353–356
22. Oka, S., and Arita, H. (1991) *J. Biol. Chem.* **266,** 9956–9960
23. Matsuzawa, A., Makoto, M., Atsumi, G., Imal, K., Prados, P., Inoue, K., and Kudo, I. (1996) *Biochem. J.* **318,** 701–709
24. O'Regan, M. H., Smith-Barbour, M., Perkins, L. M., and Phillips, J. W. (1995) *Neurosci. Lett.* **185,** 191–194
25. Lambeau, G., Barhanin, J., Schweitz, H., Qar, J., and Lazdunski, M. (1989) *J. Biol. Chem.* **264,** 11503–11510
26. Lambeau, G., Schmid-Alliana, A., Lazdunski, M., and Barhanin, J. (1990) *J. Biol. Chem.* **265,** 9526–9532
27. Lambeau, G., Ancian P., Barhanin, J., and Lazdunski, M. (1994) *J. Biol. Chem.* **269,** 1575–1578
28. Lambeau, G., Ancian, P., Barhanin, J., Nicolas, J.-P., Beiboer, S. H. W., Moinier, D., Verheij, H., and Lazdunski, M. (1995) *J. Biol. Chem.* **270,** 5534–5540
29. Piomelli, D. (1994) *Crit. Rev. Neurobiol.* **8,** 65–83
30. Shimizu, T., and Wolfe, L. S. (1990) *J. Neurochem.* **55,** 1–15
31. Bazan, N. G. (1992) in *Neurochemical Correlates of Cerebral Ischemia* (Bazan, N. G., Braquet, P., and Ginsberg, N. D., eds) pp. 321–333, Plenum Publishing Corp., New York
32. Siesjö, B. K., Ingvar, M., and Westerberg, Z. (1982) *J. Neurochem.* **39,** 796–802
33. Katsura, K., Rodriguez de Turco, E. B., Folbergrova, J., Bazan, N. G., and

Siejö, B. K. (1993) *J. Neurochem.* **61**, 1677–1684

34. Dumuis, A., Sebben, M., Haynes, J.-P., and Bockaert, J. (1988) *Nature* **336**, 68–70
35. Lazarewicz, J. W., Wroblewski, J. T., Palmer, M. E., and Costa, E. (1988) *Neuropharmacology* **27**, 765–769
36. Lazarewicz, J. W., Salinska, E., and Wroblewski, J. T. (1992) *Adv. Exp. Med. Biol.* **319**, 73–89
37. Pellerin, L., and Wolfe, L. S. (1991) *Neurochem. Res.* **16**, 983–989
38. Umemura, A., Mabe, H., Nagai, H., and Sugino, F. (1992) *J. Neurosurg.* **76**, 648–651
39. Balsinde, J., Barbour, S. E., Bianco, I. D., and Dennis, E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11060–11064
40. Balsinde, J., and Dennis, E. A. (1996) *J. Biol. Chem.* **271**, 6758–6765
41. Fonteh, A. N., Bass, D. A., Marshall, L. A., Seeds, M., Samet, J. M., and Chilton, F. H. (1994) *J. Immunol.* **152**, 5438–5446
42. Reddy, S. T., and Herschman, H. R. (1996) *J. Biol. Chem.* **271**, 186–191
43. Kini, R. M., and Evans, H. J. (1989) *Toxicon* **27**, 613–635
44. Clapp, L. E., Klette, K. L., DeCoster, M. A., Bernton, E., Petras, J. M., Dave, J. R., Laskosky, M. S., Smallridge, R. C., and Tortella, F. C. (1995) *Brain Res.* **693**, 101–111
45. Kolko, M., Rodriguez de Turco, E. B., DeCoster, M. A., and Bazan, N. G. (1996) *FASEB Abstr.*, A1255
46. DeCoster, M. A., Koening, M. L., Hunter, J. C., and Tortella, F. C. (1992) *Neuroreport* **3**, 773–776
47. Klette, K. L., DeCoster, M. A., Moreton, J. E., and Tortella, F. C. (1995) *Brain Res.* **704**, 31–41
48. DeCoster, M. A., and Yourick, D. L. (1994) *Int. J. Dev. Neurosci.* **12**, 227–233
49. Frandsen, A., and Shousboe, A. (1990) *Int. J. Dev. Neurosci.* **8**, 209–216
50. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
51. Rothman, S. M., and Olney, J. W. (1987) *Trends Neurosci.* **10**, 299–302
52. Choi, D. W. (1988) *Neuron* **1**, 623–634
53. Meldrum, B. S., and Garthwaite, J. (1990) *Trends Pharmacol. Sci.* **11**, 379–383
54. Nakatani, Y., Murakami, M., Kudo, I., and Inoue, K. (1994) *J. Immunol.* **153**, 796–803
55. Doucet, J. P., Squinto, S. P., and Bazan, N. G. (1990) *Mol. Neurobiol.* **6**, 27–55
56. Bazan, N. G., Squinto, S. P., Braquet, P., Panetta, T., and Marcheselli, V. L. (1991) *Lipids* **26**, 1236–1242
57. Bazan, N. G. (1995) *Nature* **374**, 501–502
58. Clark, G. D., Happel, L. T., Zorumski, C. F., and Bazan, N. G. (1992) *Neuron* **9**, 1211–1216
59. Kato, K., Clark, G. D., Bazan, N. G., and Zorumski, C. F. (1994) *Nature* **367**, 179–182
60. Nishizuka, Y. (1992) *Science* **258**, 607–614
61. Aveldaño, M. I., and Bazan, N. G. (1975) *Brain Res.* **100**, 99–110
62. Santos, F. F., Rodriguez de Turco, E. B., Gordon, W. C., Peyman, G. A., and Bazan, N. G. (1996) *Int. Ophthalmol.* **29**, 149–159
63. Denning, G. M., Figard, P. H., Kaduce, T. L., and Spector, A. A. (1983) *J. Lipid Res.* **24**, 933–1001
64. Spector, A. A., and Yorek, M. A. (1985) *J. Lipid Res.* **26**, 1015–1035
65. Murakami, M., Hara, N., Kudo, I., and Inoue, K. (1993) *J. Immunol.* **151**, 5675–5684
66. Upreti, G. C., and Jain, M. K. (1980) *J. Membr. Biol.* **55**, 113–121

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Lipid messengers and prostaglandin endoperoxide synthase-2 in neuronal cell death

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1. Platelet activating factor and other bioactive lipids

An important target for cerebral ischemia is phospholipids from plasma membranes of neural cells. Phospholipid molecules of membranes from neurons and glial cells store a wide variety of lipid messengers. Receptor-mediated events and changes in intracellular [Ca^{2+}], as occurs during excitatory neurotransmission and in activity-dependent synaptic plasticity, activates phospholipases that catalyze the release of bioactive moieties from phospholipids. These messengers then participate in intracellular and/or intercellular signaling pathways. Bioactive lipids have significant neurobiological actions in neurotransmitter release, synaptic plasticity, and programs of neuronal gene expression. Accordingly, contemporary research into bioactive lipids has focussed on their neurobiological significance.

Cerebral ischemia disrupts the tightly regulated events that control the production and accumulation of lipid messengers, such as free arachidonic acid, diacylglycerol and platelet-activating factor, (PAF, 1-0-alkyl-2-acyl-sn-3-phosphocholine) under physiological conditions. Rapid activation of phospholipases, particularly of phospholipase A_2 (PLA_2), occurs at the onset of cerebral ischemia (1). There are a wide variety of PLA_2s (2), and current investigations aim to define those affected by ischemia. For example, in addition to the role(s) of intracellular PLA_2s in lipid messenger formation, it has recently been discovered that a low molecular weight, secretory PLA_2 synergizes glutamate-induced neuronal damage (3). Whereas pathways leading to PLA_2 activation/release are part of normal neuronal function, ischemia-reperfusion enhances these events, overproducing PLA_2-derived lipid messengers, (e.g. enzymatically produced arachidonic acid oxygenation metabolites, non-enzymatically generated lipid peroxidation products and other reactive oxygen species), involved in neuronal damage. Among the consequences of PLA_2 activation by ischemia are alterations in mitochondrial function by the rapid increase in the brain free fatty acid pool size (e.g. uncoupling of oxidative phosphorylation from respiratory chain) and the generation of lipid messengers.

PAF is a very potent and short-lived lipid messenger. It is known to have a wide range of actions: as a mediator of inflammatory and immune responses, as a second messenger, and as a potent inducer of gene expression in neural systems. Thus, in addition to its acute roles, PAF can potentially mediate longer-term effects on cellular physiology and brain functions. In this article, the significance of PAF in synaptic function and neuronal gene expression as they relate to cerebral ischemia is discussed.

2. PAF modulates glutamate release and synaptic plasticity.

PAF enhances glutamate release in synaptically-paired rat hippocampal neurons in culture (4). The PAF analog methylcarbamyl (mc-PAF), but not biologically inactive lyso PAF, increases excitatory synaptic responses. The inhibitory neurotransmitter γ-aminobutyric acid is unaffected by mc-PAF under these conditions. The presynaptic PAF receptor antagonist BN 52021 blocks the mc-PAF-enhanced glutamate release. In addition, mc-PAF increases presynaptic glutamate release, since it does not augment the effects of exogenously added glutamate, and it evokes spontaneous synaptic responses characteristic of enhanced neurotransmitter release. Therefore, as a modulator of glutamate release, PAF participates in long-term potentiation (5), synaptic plasticity and memory formation.

3. PAF contributes to excitotoxicity by enhancing glutamate release.

Ischemia and seizures increase PAF content in brain (for reference see 1). Furthermore, brain is endowed with a variety of degradative enzymes that rapidly convert PAF to biologically inactive terpenoid extracted from the leaf of the *Ginkgo biloba* tree, which binds preferentially to the synaptosomal site (7). It is likely that this PAF binding site is the seven transmembrane PAF receptor that has been cloned by T. Shimizu, et al (for reference see 1). BN 52021 inhibits both PAF-induced glutamate release (4) and long-term potentiation (5). Moreover, this antagonist is neuroprotective in ischemia-reperfusion damage in the gerbil brain (for reference see 1). Taking these finding together, PAF, when overproduced at the synapse during ischemia, will promote enhanced glutamate release that in turn, through the activation of post-synaptic receptors, will contribute to excitotoxicity.

4. PAF is a transcriptional activator of prostaglandin endoperoxide synthase-2.

In addition to its modulatory effect on synaptic transmission and neural plasticity, PAF activates receptor-mediated immediate early gene expression. Since PAF is a phospholipid and can pass through membranes, it is rapidly taken up by cells. An intracellular binding site with very high affinity, yet pharmacologically distinct from the presynaptic site, was found in brain (7). The synthetic hetrazepine BN 50730 is selective for this intracellular site and blocks PAF-induced gene expression of the inducible form of prostaglandin G synthase in transfected cells (8).

Prostaglandin G/H synthase-2 (PGS-2, COX-2, TIS-10) catalyzes the cyclooxygenation and peroxidation of arachidonic acid into PGH_2 the precursor of biologically active prostaglandins, thromboxanes and prostacyclin. PGS-1 also catalyzes the same first committed step of the arachidonic acid cascade. PGS-2, however, is expressed in response to mitogenic and inflammatory stimuli and is encoded by an early-response gene. In contrast, PGS-1 expression is not subject to short-term regulation. Neurons in the hippocampus, as well as in a few other brain regions, are unlike other cells in that they display basal levels of PGS-2 expression (9). This expression is modulated by synaptic activity, LTP and involves the N-methyl-D-aspartate class of glutamate receptors (9,10).

PAF is a transcriptional activator of PGS-2, as PAF induces mouse PGS-2 promoter-driven luciferase activity transfected in neuroblastoma cells (NG108-15 or SH-SY5Y) and in NIH 3T3 cells. The intracellular PAF antagonist, BN 50730, inhibits PAF activation of this construct (8). Figure 1 outlines the role of PAF as a presynaptic messenger.

5. Sustained transcriptional upregulation of PGS-2 precedes kainic acid-induced neuronal damage in hippocampus.

The abundance in brain of several early-response gene transcripts shows rapid and transient increases during cerebral ischemia and after seizures. Several early-response genes encode transcription factors which in turn modulate the expression of other genes, whereas others encode inducible enzymes. The glutamate analog, kainic acid, promotes extensive neuronal damage, particularly in the hippocampus, and also induces early-response genes such as the transcription factor *zif*-268. PGS-2 is also induced under these conditions, but there are striking differences in the magnitude and duration of the induction of PGS-2 as compared with *zif*-268. PGS-2 mRNA, 2 hr after kainic acid injection, showed a 35-fold increase in hippocampus as compared with only a 5.5 fold increase in *zif*-268 (11). Also PGS-2 peak in mRNA abundance was evident at 3 hrs (71-fold increase) as compared with 1 hr for *zif*-268 (10-fold increase). *Zif*-268 mRNA time-course of changes in the hippocampus corresponds to the expected profile of early-response genes, i.e., a rapid decrease in abundance after the peak. PGS-2 on the other hand, displayed sustained upregulation for several hours after kainic acid injection (5.2 fold increase at 12 hours) (11).

6. Concluding Remarks: The platelet activating factor-prostaglandin G/H synthase-2 intracellular signaling signaling pathway and apoptosis.

A PAF-stimulated signal transduction pathway is a major component of the kainic acid-induced PGS-2 expression in hippocampus. This conclusion is based upon the finding that a) PAF induces mouse PGS-2 promoter-driven luciferase activity in transfected cells, and BN 50730 inhibits this effect (8); and b) BN 50730 (given intracerebroventricularly 15 min prior to kainic acid) inhibits kainic acid-induced PGS-2 mRNA accumulation in hippocampus by 90% (8). Both PAF (12) and PGS-2 (13) are potent mediators of the injury/inflammatory response. PAF (4,5) and PGS-2 (9, 10) are also interrelated in neuronal plasticity. The PAF transcriptional activation of PGS-2 may provide clues about novel neuronal cell death pathways. The antagonist BN 50730 was much less effective against *zif*-268 expression. In fact, the delayed hippocampal induction of PGS-2 by kainic acid precedes selective neuronal apoptosis by this agonist in this neuroanatomical region.

In cerebrovascular diseases the significance of the PLA_2-related signaling triggered by ischemia reperfusion may be part of events finely balanced between neuroprotection and neuronal cell death. The precise events that would tilt this balance toward the latter are currently being explored. It is interesting to note that PAF, being short-lived and rapidly degraded by PAF acetylhydrolase (6), is a long-term signal with consequences to neurons though PGS-2 sustained expression. PGS-2 is localized in the nuclear envelope and perinuclear endoplasmic reticulum. The overexpression of hippocampal PGS-2 during cerebral ischemia and seizures may in turn lead to the formation of neurotoxic metabolites (e.g. superoxide). Current investigations aim to determine whether or not other messengers cooperate to enhance neuronal damage (e.g. nitric oxide) and the possible involvement of astrocytes and microglial cells. Further understanding of these potentially neurotoxic events involving lipid messengers and PGS-2 will permit the identification of new strategies and define therapeutic windows for the management of cerebrovascular diseases.

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References

1. N.G. Bazan, E.B. Rodriguez de Turco, G. Allan, Mediators of injury in neurotrauma: Intracellular signal transduction and gene expression. J. Neurotrauma 12, 789-911 (1995).

2. E.A. Dennis, Diversity of group types, regulation and function of phospholipase A_2. J. Biol. Chem. 269, 13057-13060 (1994).

3. M. Kolko, M.A. DeCoster, E.B. Rodriguez de Turco, N.G., Synergy by secretory phospholipase A_2 and glutamate on inducing cell death and sustained arachidonic acid metabolic changes in primary cortical neuronal cultures. J. Biol. Chem. (in press).

4. G.D. Clark, L.T. Happel, C.F. Zorumski, Bazan NG, Enhancement of hippocampal excitatory synaptic transmission by platelet-activating factor. Neuron 9, 1211-1216 (1992).

5. K. Kato, G.D. Clark, N.G. Bazan, C.F. Zorumski, Platelet activating factor as a potential retrograde messenger in Ca^1 hippocampal long-term potentiation. Nature 367, 175-179 (1994).

6. N.G. Bazan, Inflammation: A signal terminator. Nature 374, 501-502 (1995).

7. V.L. Marcheselli, M. Rossowska, M.T. Domingo, P. Braquet, N.G. Bazan, Distinct platelet-activating factor binding sites in synaptic endings and in intracellular membranes of rat cerebral cortex. J. Biol. Chem. 265, 9140-9145 (1990).

8. N.G. Bazan, B.S. Fletcher, H.R. Herschman, P.K. Mukherjee, Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene. Proc. Natl. Acad. Sci. 91, 5252-5256 (1994).

9. K. Yamagata, K.I. Andreasson, W.E. Kaufmann, C.A. Barnes, P.F. Worley, Expression of

a mitogen-inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids. Neuron 11, 371-386 (1993).

10. W.E. Kaufmann, P.F. Worley, J. Pegg, M. Bremer, P. Isakson, COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. Proc. Natl. Acad. Sci. 93, 2317-2321 (1996).

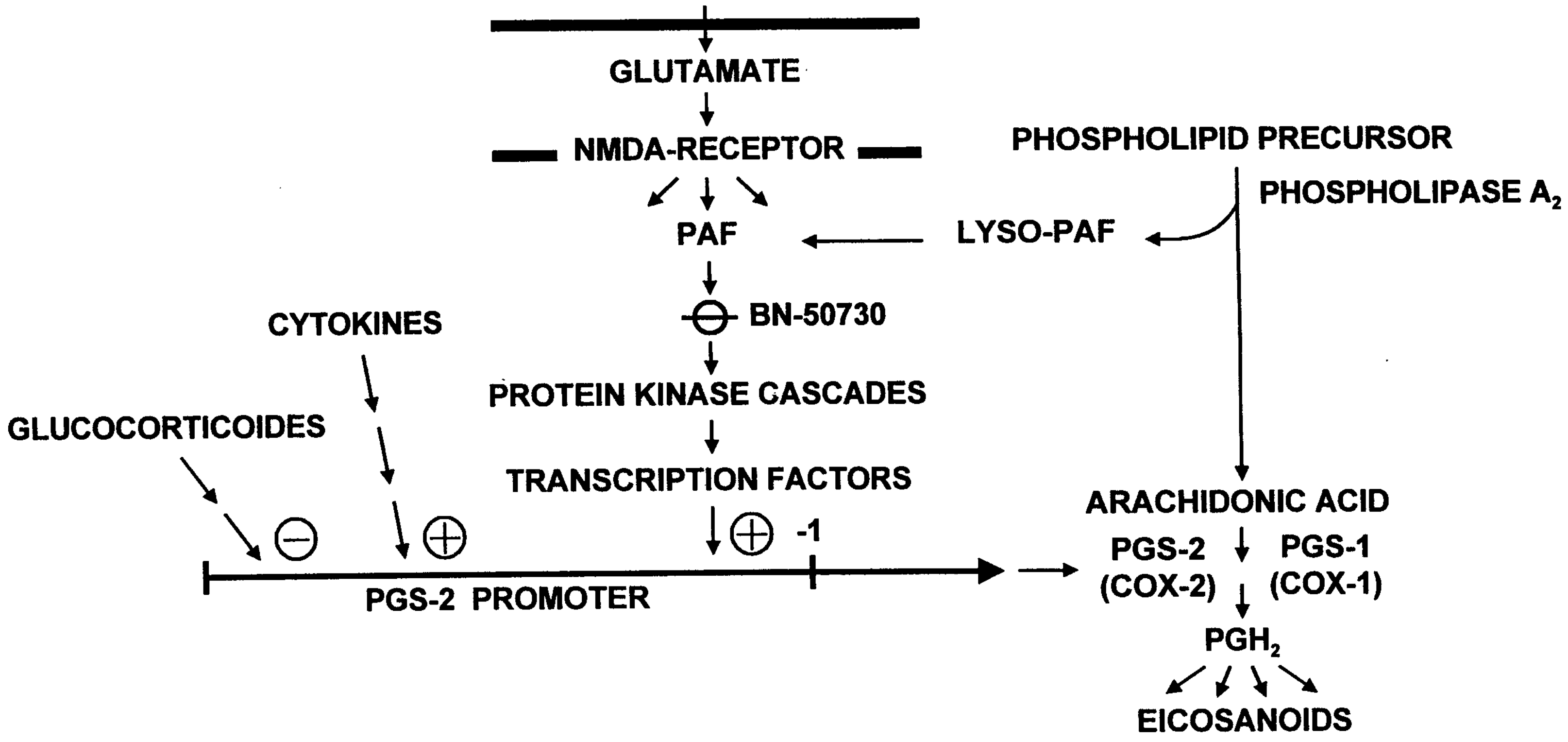
11. V.L. Marcheselli, N.G. Bazan, Sustained induction of prostaglandin endoperoxide synthase-2 by seizures in hippocampus. Inhibition by a platelet-activating factor antagonist. J. Biol. Chem. 271, 24794-24799 (1996).

12. S.M. Prescott, G.A. Zimmerman, T.M. McIntyre, Platelet-activating factor. J. Biol. Chem. 265, 17381-17384 (1990).

13. N.G. Bazan, J. Botting, J.R. Vane (Eds.), New Targets in Inflammation: Inhibitors of COX-2 or Adhesion Molecules. William Harvey Press and Kluwer Academic Publishers, United Kingdom (1996).

14. N.G. Bazan and G. Allan, Platelet-activating factor is both a modulator of synaptic function and a mediator of cerebral injury and inflammation. Adv. in Neurol. 71, 475-484 (1996).

Figure 1: Ischemia-triggered signaling events linking synapse activation and PGS-2 gene expression in neurons. NMDA-receptor activation by glutamate leads to phospholipase A_2 activation and the generation of PAF and of arachidonic acid. PAF is synthesized through other metabolic routes as well (6). PAF activates PGS-2 gene expression through a BN-50730-sensitive intracellular site, protein kinase cascades and transcription factors. The PGS-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). PGS-2 protein then catalyzes the conversion of arachidonic acid into PGH_2, the precursor of eicosanoids. Constitutive PGS-1 also catalyzes this metabolic step.


GLUTAMATE
NMDA-RECEPTOR
PHOSPHOLIPID PRECURSOR
PHOSPHOLIPASE A_2
PAF
LYSO-PAF
BN-50730
CYTOKINES
PROTEIN KINASE CASCADES
GLUCOCORTICOIDES
TRANSCRIPTION FACTORS
⊖
⊕
⊕
-1
PGS-2 PROMOTER
ARACHIDONIC ACID
PGS-2
(COX-2)
PGS-1
(COX-1)
PGH_2
EICOSANOIDS

Synaptic Messengers, Inflammatory Mediators, and Neuronal Plasticity in Cerebral Ischemia.

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Key words: COX-2, LTP, PAF, Prostaglandins

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Summary

Cerebral ischemia activates the production of both endogenous and exogenous mediators of the inflammatory response. Using the middle cerebral artery occlusion suture model in the rat, we have found that occlusion, followed by reperfusion, leads to a remarkable induction of cyclooxygenase-2, both in the infarcted area as well as in the penumbra, occurring 6 hours after 2 hours of ischemia, followed by three hours of reperfusion. COX-1, the constitutive enzyme, does not display changes under these conditions. Recent studies in our laboratory have also shown that in a kainic acid-induced brain damage model, COX-2 induction occurs at the transcriptional level, since nuclear runs on assays in isolated nuclei from the hippocampus as a function of time after injury matched the profile of changes in COX-2 mRNA abundance. Moreover, platelet-activating factor is an activator of COX-2 transcription in transfected cells and PAF antagonists in vivo block the injury-induced COX-2 expression. Therefore PLA_2 activation and PAF generation in cerebral ischemia link the formation of prostaglandins through COX-2 induction. These signaling pathways may be transneuronally controlled through excitatory amino acid neurotransmitters. It is hypothesized that PLA_2 activation, PAF accumulation and COX-2 induction are reduced during the development of enhanced tolerance to ischemic injury.

1. Introduction

The earliest biochemical events involving degradation of membrane phospholipids in cerebral ischemia and seizures are an accumulation of free arachidonic acids (Bazan, 1970), eicosanoids (Birkle and Bazan, 1987) and platelet activating factor (PAF, 1-O-alkyl-2-acetyl-glycero-3-phosphocholine) (Kumar et al, 1988). The stimulus for the release of these bioactive mediators is an activation of phospholipases A_2 (Birkle and Bazan, 1987) and C (Aveldano and Bazan, 1975). The release represents an overactivation of the normal physiological processes by which these lipid mediators are synthesized, and exemplifies how certain pools of excitable membrane phospholipids act as reservoirs for the stimulus-elicited release of lipid second messengers. One such reservoir is a small, but metabolically active pool of 1-O-alkyl-2-arachidonoyl-glycero-3-phosphocholine (alkylacyl-GPC) that upon hydrolysis by PLA_2 yields both arachidonic acid and lyso PAF, a direct precursor of PAF. Alkylacyl-GPC is a substrate for a brain cytosolic PLA_2, activated and translocated to the membrane by intracellular calcium (Yoshijara and Watanabe, 1990; Bonventre et al, 1993). Lyso-PAF is transacetylated to yield biologically active PAF via the remodeling pathway of PAF synthesis. There are at least two alternative pathways by which PAF can be generated: via the *de novo* synthesis route and via a CoA-independent transacylase. While the contribution of these pathways to PAF synthesis during neuronal stimulation has yet to be fully evaluated, the rise in intracellular calcium which stimulates the remodeling pathway also inhibits the phosphocholine transferase required in the *de novo* route (Francesangeli and Goracci, 1989).

There are high-affinity PAF binding sites in the brain (Marcheselli et al, 1989) which are kinetically and pharmacologically distinct in synaptosomes and microsomes. The two sites differ in their sensitivity to different PAF antagonists. The *Ginkgo bilbo,* a tree-derived BN 52021, binds preferentially to the synaptosomal site (Marcheselli et al, 1989), and the synthetic hetrazepine BN 50730, which shows specificity for the microsomal site. The synaptosomal site appears to correspond to the cloned PAF receptor (Honda et al, 1991; Kunz et al, 1992; Sugimoto et al, 1992) of the seven membrane-spanning domain, G protein-coupled receptor superfamily. The identity of the BN 50730-sensitive receptor requires elucidation, but it has a specific role in PAF-induced primary-early gene expression in brain in that it differs at least in its signal transduction coupling from the synaptosomal receptor.

While the pathophysiological effects of PAF on inflammatory and immune responses in many tissues and organs have been well-documented (for review Prescott et al, 1990), it is now becoming apparent that, via its modulation of glutamate release, PAF can also have profound effects on excitatory neurotransmission, excitotoxic neuronal damage and synaptic plasticity.

2. PAF Activates Gene Transcription of the Inducible Prostaglandin Synthase and of Other Early-Response Genes

Physiological and pathological events such as NMDA receptor activation, long-term potentiation, ischemia, and seizures initiate transcription of genes encoding transcription factors, and thus have the potential to initiate cascades of gene expression (Doucet and Bazan, 1993; Kogure and Kato, 1993; Kiessling and Gass, 1993)

PAF is a potential mediator in the coupling of short-lived cell signaling events and long-term genomic responses because it is stimulates immediate early-response gene expression in neuronal and other cells in culture. Furthermore, the use of specific PAF antagonists blocks gene expression in animal models of brain trauma. PAF rapidly and transiently augments, via transcriptional activation, levels of the c-*fos* and c-*jun* transcription factor mRNAs in a neural cell line (Squinto et al, 1989). 5′ deletion mutagenesis studies of the c-*fos* promoter show that the calcium-response element is necessary for the PAF-induced response. Expression of the mRNAs for c-fos and the zinc finger transcription factor *zif*268 induced by addition of PAF to rat astroglia (Dell' Albani et al, 1993). PAF also induces early response gene expression in immune, inflammatory and other cells, including the heparin-binding epidermal growth factor in monocytes (Pan et al, 1989), *c-fos* and egr-2 in lymphoblastoid cell lines (Mazer et al, 1991), the transcription factor NF-kappa B and immunoglobulins in human B cell lines

(Smith and Shearer, 1994), *c-fos* and TIS 1 in A-431 epidermoid carcinoma cells (Tripathy et al, 1991), and *c-fos* and *c-jun* i rabbit corneal epithelium (Bazan et al, 1993).

A role for PAF in the induction of early response gene expression in cerebral ischemia or neurotrauma is implied by the inhibitory effects of the intracellular PAF receptor antagonist BN 50730. A single electroconvulsive shock rapidly elevates *c-fos* and *zif*268 mRNA levels in the rat hippocampus and cerebral cortex, an effect that is partially blocked by pretreatment of the animals with an intraperitoneal (systemic) or intracerebroventricular (localized) injection of BN 50730 in the absence of overt changes in seizure activity (Marcheselli and Bazan, 1994).

PAF can also induce the inducible form of prostaglandin synthase (PGHS-2, COX 2). Prostaglandin synthase catalyzes the rate-limiting step in the oxygenation of arachidonic acid to PGH_2, the precursor of prostaglandins, and is therefore an important control point for the regulation of lipid second messenger synthesis. The constitutive isoform, PGHS-1, appears to be responsible for prostaglandin synthesis under physiological conditions, while the inducible isoform, PGHS-2, which is encoded by an early-response gene (Kujubu et al, 1991; O'Banion et al, 1991), is responsible for the augmentation of prostaglandin synthesis during pathophysiological responses. However, PGHS-2 is expressed constitutively, but is still inducible, in post-natal rat brain (Yamagata et al, 1993) and rabbit corneal epithelium (Tao et al, 1995). It has recently been shown that PGHS-1 is localized mainly in the endoplasmic reticulum in mouse 3T3 fibroblasts, while PGHS-2 is concentrated in the nuclear envelope (Morita et al, 1995). This suggests that PGHS-2 induction may direct prostaglandin action to the nucleus (Goetzl et al, 1995). PGHS-2 is inducible by a number of mitogens, growth factors and cytokines, and its induction is inhibited by glucocorticoids (Herschman, 1994).

PAF can induce PGHS-2 expression in non-neuronal cells and in the brain. When constructs are transfected into cells using the calcium phosphate-calcium coprecipitation procedure, in the presence of retinoic acid there is dose-dependent (1-50nM) PAF induced expression of the reporter, driven by the proximal 371bp of the mouse PGHS-2 promoter (Bazan et al, 1994). There is also activity from a 963bp promoter fragment although less, suggesting the presence of inhibitory sequences upstream from -371. The effect is rapid, with some activity as soon as 15 or 45 min of incubation with the ligands, suggesting the involvement of pre-existing transcription factors. Preincubation of the cells with the intracellular PAF antagonist BN 50730 further supports this effect as being a specific receptor mediated phenomenon. The use of constructs with 5' deletions of this promoter narrows down the main "PAF-responsive" region of the promoter to between -371 and -300; deletion of this region lowers PAF induction from 31 times to 4.1 times control levels.

In two experimental models of rat brain trauma, seizures and cryogenically-induced vasogenic edema, PGHS-2 mRNA and protein are induced while PGHS-1 expression remains unaltered. Pretreatment of the animals with BN50730 significantly reduces PGHS-2 induction (Marcheselli and Bazan, 1996). Unlike the expression of other immediate-early genes in these models, which typically reach peak expression by 1 hour after stimulus and return to basal levels within 3-4 hours, PGHS-2 expression reaches a peak 6 hours after stimulus and remains elevated at 24 hours. BN50730 is able to inhibit this induction more effectively than dexamethasone, which is a synthetic glucocorticoid that is a potent inhibitor of PGHS-2 induction in many cells and tissues. This would suggest that the PAF antagonist and the glucocorticoid inhibit gene expression via distinct mechanisms.

3. Conclusion: Potential Significance of Bioactive Lipids to Maturation Phenomenon in Cerebral Ischemia

PAF has multiple effects on excitatory neurotransmission and neural gene expression, and is involved in the modulation of synaptic function in physiological and pathophysiological situations. PLA_2, PAF and COX-2 induction may represent a signaling pathway transneuronally modulated by excitatory amino acid neurotransmitters. The neuroprotection by presynaptically-active PAF receptor antagonists in animal models of cerebral ischemia (Panetta et al, 1988; Gilboe et al, 1991; Prehn et al, 1993) may be due in part to their inhibitory effects on excitotoxic neuronal damage, as well as to their inhibition of PAF-induced synthesis of inflammatory mediators. In addition, the.intracellular PAF antagonist BN 50730 inhibits kindling epileptogenesis (Moises et al, 1994). In figure 2, PAF involvement in pathways of neuronal plasticity or apoptosis is depicted. The induction of cascades of gene expression, and also in inflammatory events, by stimulation of prostaglandin synthesis. Inducible prostaglandin synthase in ischemic injury and during reperfusion following cerebral ischemia, along with potential effects of prostaglandins as modulators of cerebral circulation, are involved in vasodilation and enhancement of cerebral blood flow (Hallenbeck and Furlow, 1979). Conversely, the breakdown of the blood-brain barrier and infiltration of inflammatory cells during cerebral edema might allow lipid mediators of extra neural origin to exert their effects on neuronal cells.

Because of the significance of a) PAF in synaptic plasticity, long-term potentiation , and the formation of certain forms of memory; b) PAF in excitotoxicity, and; c) COX-2 both in plasticity. As well as in the injury-inflammatory response, we hypothesized that these events play a central role in the enhanced tolerance to ischemic injury. Thus, it is predicted that PLA_2 activation, PAF accumulation, and COX-2 induction should be reduced under these conditions. This hypothesis remains to be tested.

References

Aveldaño, M. I. and Bazan, N.G. (1975) Rapid production of diacylglycerols enriched in 20:4 and stearate during early brain ischemia. J. Neurochem. 25, 919-920.

Bazan, H. E. P., Tao, Y. and Bazan, N.G. (1993) Platelet-activating factor induces collagenase expression in corneal epithelial cells. Proc. Natl. Acad. Sci. USA. 90, 8678-8682.

Bazan NG. (1970) Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. Biochim. Biophys. Acta. 218, 1-10.

Bazan, N. G., Fletcher, B. S., Herschman, H. R. and Mukherjee, P. K. (1994) Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene. Proc. Natl. Acad. Sci. USA. 91, 5252-5256.

Birkle, D.L. and Bazan, N.G. (1987) Effect of bicuculline-induced status epilepticus on prostaglandins and hydroxyeicosatetraenoic acids in rat brain subcellular fractions. J. Neurochem. 48, 1768-1778.

Bonventre, J.V. and Koroshetz, W.J. (1993) Phospholipase A_2 (PLA_2) activity in gerbil brain: characterization of cytosolic and membrane-associated forms and effects of ischemia and reperfusion on enzymatic activity. J. Lipid Mediat. 6, 457-471.

Dell' Albani, P., Condorelli, D. F., Mudo, G., Amico, C., Bindoni, M. and Belluardo N. (1993) Platelet-activating factor and its methoxy analogue ET-18-OCH3 stimulate immediate-early gene expression in rat astroglial cultures. Neurochem. Int. 22, 567-574

Doucet, J. P. and Bazan, N. G. (1993) Excitable membranes, lipid messengers and immediated-early genes. Alteration of signal transduction in neuromodulation and neurotrauma. Mol. Neurobiol. 6, 407-424.

Francescangeli, E. and Goracci, G. (1989) The *de novo* biosynthesis of platelet-activating factor in rat brain. Biochem. Biophys. Res. Commun. 161, 107-112.

Futaki, N., Takahashi, S., Yokoyama, M., Arai, I., Higuchi, S. and Otomo, S. (1994) NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity *in vitro*. Prostaglandins 47, 55-59.

Gilboe, D. D., Kintner, D., Fitzpatrick, J. H., Emoto, S. E., Esanu, A., Braquet P. G., and Bazan N. G. (1991) Recovery of postischemic brain metabolism and function following treatment with a free radical scavenger and platelet activating factor. J. Neurochem. 56, 311-319.

Goetzl, E. J., An, S. and Smith, W. L. (1995) Specificity of expression of eicosanoid mediators in normal physiology and human disease. FASEB J. 9, 1051-1058.

Hallenbeck, J M. and Furlow T. W. (1979) Prostaglandin I_2 and indomethacin prevent inhibition of post-ischemic brain reperfusion in the dog. Stroke 10, 629-637.

Herschman, H. R. (1994) Regulation of prostaglandin synthase-1 and prostaglandin synthase-2. Cancer Metastasis Rev. 13, 241-256.

Honda, Z., Nakamura, M., Miki, I., Minami, M., Wantabe, T., Seyama, Y., Okado, H., Toh, H., Ito, K., Miyamoto, T. and Shimizu, T. (1991) Cloning by functional expression of platelet-activating factor receptor from guinea-pig lung. Nature 394, 342-346.

Kato, K., Clark, G. D., Bazan, N. G. And Zormuski, C. F. (1994) Platelet-activating factor as a potential retrograde messenger in CA1 hippocampal long-term potentiation. *Nature* **367**, 179-182.

Kiessling, M. and Gass, P. (1993) Immediate early gene expression in experimental epilepsy.

Kiessling, M. and Gass, P. (1993) Immediate early gene expression in experimental epilepsy. Brain Pathol. 3, 381-393.

Kogure, K. and Kato, H. (1993) Altered gene expression in cerebral ischemia. Stroke 24, 2121-2127.

Kujubu, D. A., Fletcher, B. S., Varnum, B. C., Lim, R.W. and Herschman, H. R. (1991) TIS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J. Biol. Chem. 266, 12866-12872.

Kumar, R., Harvey, S., Kester, N., Hanahan, D. and Olson, M. (1988) Production and effects of platelet-activating factor in the rat brain. Biochim Biophys Acta. 963, 375-383.

Kunz, D., Gerard, N. P. and Gerard, C. (1992) The human leukocyte PAF receptor. cDNA cloning, cell surface expression and construction of a novel epitopr-bearing analog. J. Biol. Chem. 267, 9101-9106.

Marcheselli, V.L., Rossowska, M., Domingo, M.T., Braquet, P. and Bazan, N.G. (1990) Distinct platelet-activating factor binding sites in synaptic endings and in intracellular membranes of rat cerebral cortex. J. Biol. Chem. 265, 9140-9145.

Marcheselli, V.L. and Bazan, N.G. (1994) Platelet-activating factor is a messenger in the electroconvulsive shock-induced transcriptional activation of c-*fos* and *zif*-268 in hippocampus. J. Neurosci. Res. 37, 54-61.

Marcheselli, V.L. and Bazan, N.G. (1996) Sustained Induction of Prostaglandin Endoperoxide Synthase-2 by Seizures in Hippocampus: Inhibition by a Platelet-Activating Factor Antagonist. J. Biol. Chem. (In press).

Mazer, B., Domenico, J., Sawami, H. and Gelfand E W. (1991) Platelet-activating factor induces an increase in intracellular calcium and expression of regularity genes in human B lymphoblastoma cells. J Immunol. 146, 1914-1920.

Moises, J., Allan, G., Marcheselli V. L., Mahandere, V., and Bazan N G. (1994) A PAF antagonist inhibits the expression o hippocampal immediate early genes in a kindling model of epileptogenesis. Epilepsia 35, 139.

Morita, I., Schindler, M., Regier, M. K., Otto, J. C., Hori, T. DeWitt, D. L. and Smith W L. (1995) Different intracellular locations for prostaglandin endoperoxide H synthase-1 and -2. J. Biol. Chem. 270, 10902-10908.

O'Banion, M. K., Sadowski, H. B., Winn, V. and Young, D. A. (1991) A serum- and glucocorticoid-regulated 4-kilobase mRNA encodes a cycooxygenase-related protein. J. Biol. Chem. 266, 23261-23267.

Pan, Z., Kravchenko, V. V. and Ye, R. D. (1995) Platelet activating factor stimulates transcription of the heparin-binding epidermal growth factor-like growth factor in monocytes. Correlation with an increased kappa B binding activity. J. Biol. Chem. 270, 7787-7790.

Panetta, T., Marcheselli, V.L., Braquet, P., Spinnewyn, B. and Bazan, N.G. (1987) Effects of a platelet-activating factor antagonist (BN 52021) on free fatty acids, diacylglycerols, polyphosphoinositides and blood flow in the gerbil brain: Inhibition of ischemia-reperfusion induced cerebral injury. Biochem. Biophys. Res. Commun. 149, 580-587.

Prehn, J. H. M. and Krieglstein, J. (1993) Platelet-activating factor antagonists reduce excitotoxic damage in cultured neurons from embryonic chick telencephalon and protect the rat hippocampus and neocortex from ischemic injury *in vivo*. J. Neurosci. Res. 34, 179-188.

Prescott, S.M., Zimmerman. G.A. and McIntyre, T.M. (1990) Platelet-activating factor. J. Biol. Chem. 265, 17381-17384.

Smith, C. S. and Shearer W. T. (1994) Activation of NF-kappa B and immunoglobulin expression in response to platelet-activating factor in a human B cell line. Cell. Immunol. 155, 292-303.

Squinto, S.P., Block, A.L., Braquet, P. and Bazan, N.G. (1989) Platelet-activating factor stimulates a Fos/Jun/AP-1 transcriptional signaling system in human neuroblastoma cells. J. Neurosci. Res. 24, 558-566.

Sugimoto, T., Tsuchimochi, H., McGregor, C. G., Mutoh, H., Shimizu, T. and Kurachi Y. (1992) Molecular cloning and characterization of the platelet-activating factor receptor from human heart. Biochem. Biophys. Res. Commun. 189, 617-624.

Tao, Y, Bazan, N.G., Bazan, H.E.P. (1995) Platelet-activating factor (PAF) increases prostaglandin synthase-2 (COX-2) gene expression in corneal epithelium. Arvo abstract 36, S573.

Tripathi, Y., Kandala, J., Guntaka, R., Lim, R. and Shukla, S. (1991) Platelet-activating factor induces expression of early response genes c-*fos* and TIS-1 in human epidermoid carcinoma-432 cells. Life. Sci. 49, 1761-1767.

Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A. and Worley, P. F. (1993) Expression of a mitogen-inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids. Neuron 11, 371-386.

Yoshijara, Y. and Watanabe, Y. (1990) Translocation of phospholipase A_2 from cytosol to membranes in rat brain induced by calcium ions. Biochem. Biophys. Res. Commun. 170, 484-490.

Figure legends.

Figure 1. **Transneuronal signaling and bioactive lipids.** PAF enhances glutamate release and is involved in long-term potentiation. A depolarizing stimulus at the nerve ending triggers glutamate release. Glutamate occupancy of the postsynaptic NMDA receptor raises intracellular calcium in the postsynaptic neuron. Activation and membrane translocation of the cytoplasmic PLA_2 results in the release of free arachidonic acid and PAF. While these bioactive lipids have very short biological half lives, upon repeated stimulus sufficient PAF accumulates to diffuse back across the synaptic cleft (Kato, et al, 1994). PAF binds to its presynaptic receptor and enhances glutamate excytotosis by an as yet undefined mechanism. The free arachidonic acid released by PLA_2 activity can further enhance neurotransmission by acting on the NMDA receptor. During synaptic plasticity events, sufficient PAF might accumulate to activate the gene expression which is probably involved in long-term alterations of synaptic function.

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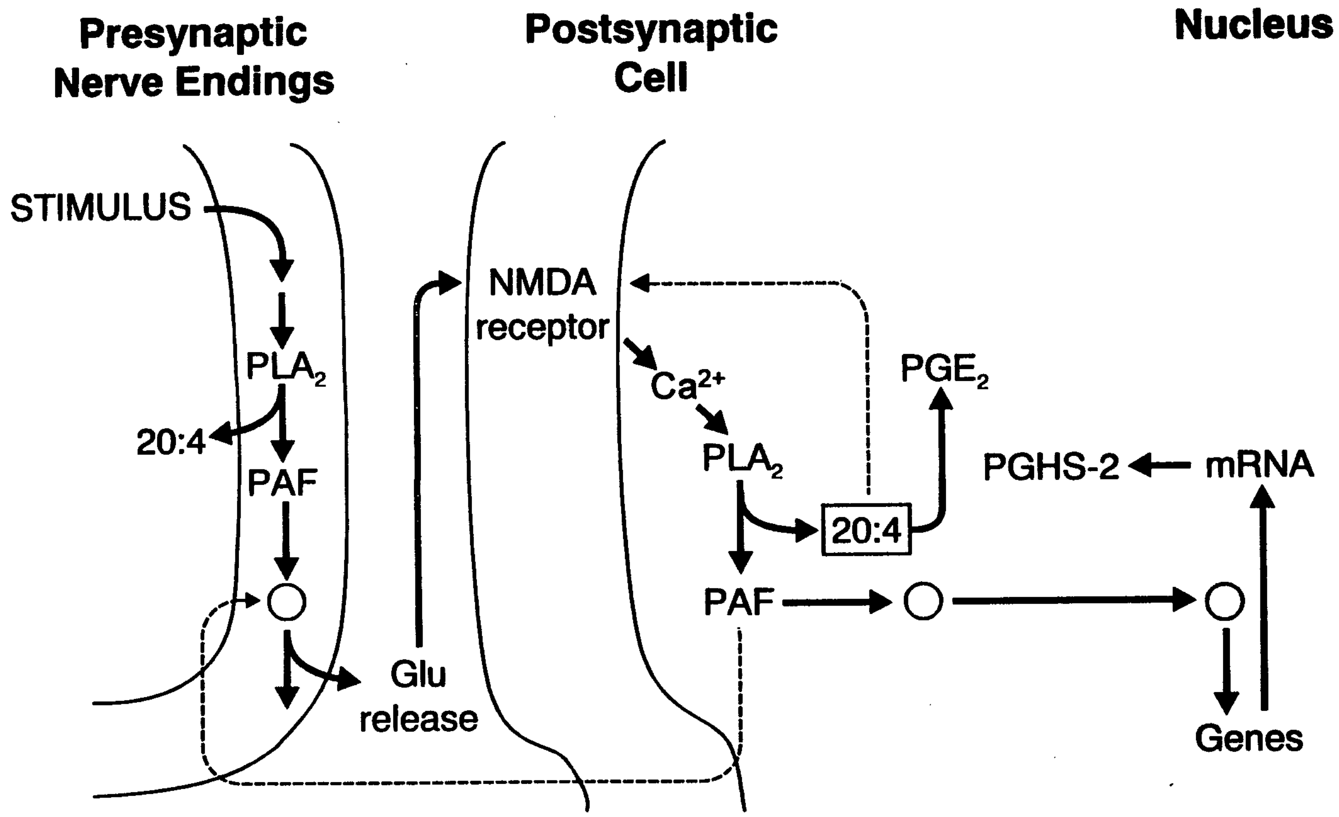
Presynaptic Nerve Endings
Postsynaptic Cell
Nucleus
STIMULUS
PLA2
20:4
PAF
Glu release
NMDA receptor
Ca2+
PLA2
20:4
PGE2
PAF
PGHS-2
mRNA
Genes

BAZN1727.DS4

The Inflammatory Mediator Platelet-Activating Factor and the Inducible Prostaglandin Synthase (COX-2) Gene in CNS Diseases

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Short Title: PAF and COX-2

Abstract

Platelet-activating factor (PAF, 1-0-alkyl-sn-2-acetyl-3-phosphocholine), the most potent biologically active lipid known, is involved in the injury/inflammatory response in many cells. In brain, PAF is rapidly produced at the onset of ischemia and seizures. PAF, the only bioactive phospholipid to have a cloned receptor, also elicits actions through an intracellular site. Physiologically, PAF modulates glutamate release, long-term potentiation (LTP) and memory formation. Furthermore, PAF is a transcriptional activator of COX-2. Interestingly, COX-2, as well as PAF, is involved in both synaptic plasticity (e.g., LTP) and in the injury/inflammatory response. Unlike in other cells, however, COX-2 is constitutively expressed in neurons at low levels.

During kainic acid-induced epileptogenesis, we find a sustained upregulation of COX-2 in hippocampus, several fold greater than another early response gene, *zif*-268. Kainic acid-induced COX-2 overexpression is mainly due to transcriptional activation and precedes hippocampal neuronal apoptosis under these conditions. Pretreatment of animals with the intracellular PAF antagonist BN 50730 strongly attenuates COX-2 induction by kainic acid. Currently, we are testing the hypothesis that the overexpression of the PAF-COX-2 pathway leads to neuronal cell apoptosis in several CNS disease models. Although it is often stated that the inflammatory response is not associated with apoptotic cell death, PAF and COX-2 appear to act as neuron injury messengers without reflecting classical "inflammatory" features to the entire tissue.

Key words: COX-2, PAF, kainic acid, apoptosis, hippocampus, gene expression, neuroprotection

Introduction

Neuronal and glial cell membranes store a wide variety of lipid messengers as part of phospholipid molecules. Receptor-mediated events and changes in intracellular [Ca^{2+}], as occurs during excitatory neurotransmission and in activity-dependent synaptic plasticity, activates phospholipases that catalyze the release of bioactive moieties from membrane phospholipid. These messengers then participate in intracellular and/or intercellular signaling pathways. Bioactive lipids have significant neurobiological actions in neurotransmitter release, synaptic plasticity, and programs of neuronal gene expression. Accordingly, much of contemporary research into bioactive lipids has focussed on their neurobiological significance.

Stroke, neurotrauma, epileptic brain damage and, likely, neurodegenerative diseases (e.g., Alzheimer's) disrupt the tightly regulated enzymes that control the production and accumulation of lipid messengers, such as free arachidonic acid, diacylglycerol and platelet-activating factor, (PAF, 1-0-alkyl-2-acyl-sn-3-phosphocholine) under physiological conditions. Rapid activation of phospholipases, particularly of phospholipase A_2 (PLA_2), occurs at the onset of cerebral ischemia and seizures (1). There are a wide variety of PLA_2s (2), and current investigations aim to define those affected by different pathological conditions. For example, in addition to the role(s) of intracellular PLA_2s in lipid messenger formation, it has recently been discovered that a low molecular weight, secretory PLA_2 synergizes glutamate-induced neuronal damage (3). Therefore, a synaptic secretory PLA_2 may also be a target in these diseases. Whereas pathways leading to PLA_2 activation/release are part of normal neuronal function, ischemia-reperfusion and other pathological conditions enhance these events, overproducing PLA_2-derived lipid messengers involved in neuronal damage (e.g. enzymatically produced arachidonic acid oxygenation metabolites, non-enzymatically generated lipid peroxidation products and other reactive oxygen species, PAF). For example, among the consequences of PLA_2 activation by ischemia are alterations in mitochondrial function by the rapid increase in the brain free fatty acid pool size that leads to the uncoupling of oxidative phosphorylation from respiratory chain. The major consequence is the pathological accumulation of lipid messengers.

PAF is a very potent and short-lived lipid messenger. It is known to have a wide range of actions: as a mediator of inflammatory and immune responses, as a second messenger, and as a potent inducer of gene expression in neural systems. Thus, in addition to its acute roles, PAF can potentially mediate longer-term effects on cellular physiology and brain functions. Furthermore, the early response gene, prostaglandin endoperoxide synthase-2 (PGS-2, COX-2, TIS-10) performs a dual function similar to PAF. Prostaglandin G/H synthase-2 catalyzes the cyclooxygenation and peroxidation of arachidonic acid into PGH_2, the precursor of biologically active prostaglandins, thromboxanes and prostacyclin. PGS-1 also catalyzes the same first committed step of the arachidonic acid cascade. PGS-2, however, is expressed in response to mitogenic and inflammatory stimuli. In contrast, PGS-1 expression is not subject to short-term regulation. Neurons in the hippocampus, as well as in a few other brain regions, are unlike other cells in that they display basal levels of PGS-2 expression (4). This expression is modulated by synaptic activity, LTP and involves the N-methyl-D-aspartate class of glutamate receptors (4,5). In pathological environments present in seizures, ischemia and neurodegenerative diseases such as Alzheimer's disease, an increased expression of PGS-2 is observed. Additionally, since PAF is a transcriptional activator of PGS-2 (6), both may be linked as a signaling system in neuronal responses to synaptic activation and in CNS diseases. Moreover, PGS-2 is expressed in neurons

(4) and PAF elicits neuronal plasticity actions (7-9). In this chapter, the significance of PAF and PGS-2 as inflammatory mediators in the CNS is discussed.

PAF modulates synaptic plasticity, and in pathological conditions, contributes to excitotoxicity by enhancing glutamate release.

PAF enhances glutamate release in synaptically-paired rat hippocampal neurons in culture (8). The PAF analog methylcarbamyl (mc-PAF), but not biologically inactive lyso PAF, increases excitatory synaptic responses. The inhibitory neurotransmitter γ-aminobutyric acid is unaffected by mc-PAF under these conditions. The presynaptic PAF receptor antagonist BN 52021 blocks the mc-PAF-enhanced glutamate release. In addition, mc-PAF increases presynaptic glutamate release, since it does not augment the effects of exogenously added glutamate, and it evokes spontaneous synaptic responses characteristic of enhanced neurotransmitter release. Therefore, as a modulator of glutamate release, PAF participates in long-term potentiation (9), synaptic plasticity and memory formation.

Ischemia and seizures increase PAF content in brain (for reference see 1). Furthermore, brain is endowed with a variety of degradative enzymes that rapidly convert PAF to biologically inactive lyso PAF (10). Presynaptic membranes display PAF binding that can be displaced by BN 52021, a terpenoid extracted from the leaf of the *Ginkgo biloba* tree, which binds preferentially to the synaptosomal site (11). It is likely that this PAF binding site is the seven transmembrane PAF receptor that has been cloned (1). BN 52021 inhibits both PAF-induced glutamate release (4) and long-term potentiation (9). Moreover, this antagonist is neuroprotective in ischemia-reperfusion damage in the gerbil brain (for reference see 1). Taking these findings together, PAF, when overproduced at the synapse during ischemia, will promote enhanced glutamate release that in turn, through the activation of post-synaptic receptors, will contribute to excitotoxicity.

PAF is a transcriptional activator of prostaglandin endoperoxide synthase-2.

In addition to its modulatory effect on synaptic transmission and neural plasticity, PAF activates receptor-mediated immediate early gene expression (12-14). Since PAF is a phospholipid and can pass through membranes, it is rapidly taken up by cells. An intracellular binding site with very high affinity, yet pharmacologically distinct from the presynaptic site, was found in brain (11). The synthetic hetrazepine BN 50730 is selective for this intracellular site and blocks PAF-induced gene expression of PGS-2 in transfected cells (6).

PAF is a transcriptional activator of PGS-2, as PAF induces mouse PGS-2 promoter-driven luciferase activity transfected in neuroblastoma cells (NG108-15 or SH-SY5Y) and in NIH 3T3 cells. The intracellular PAF antagonist, BN 50730, inhibits PAF activation of this construct (6). Figure 1 outlines the role of PAF as a presynaptic messenger.

Sustained transcriptional upregulation of PGS-2 precedes kainic acid-induced neuronal damage in hippocampus.

The abundance in brain of several early-response gene transcripts shows rapid and transient increases during cerebral ischemia and after seizures (15, 16). Several early-response genes encode transcription factors which in turn modulate the expression of other genes, whereas others encode inducible enzymes. The glutamate analog, kainic acid, promotes extensive neuronal damage, particularly in the hippocampus (17), and also induces early-response genes such as the transcription factor *zif*-268 (16). PGS-2 is also induced under these conditions, but there are striking differences in the magnitude and duration of the induction of PGS-2 as compared with *zif*-268. The PGS-2 peak in mRNA abundance was evident at 3 hrs (71-fold increase) as compared to 1 hr for *zif*-268 (10-fold increase). *Zif*-268 mRNA time-course of changes in the hippocampus corresponds to the expected profile of early-response genes, i.e., a rapid decrease in abundance after the peak is apparent. PGS-2, on the other hand, displayed sustained upregulation for several hours after kainic acid injection (5.2 fold increase at 12 hours) (18). The increased PGS-2 mRNA abundance in hippocampus after kainic acid may be due to enhanced transcription, increased availability of factors that prolong mRNA half-life (e.g., PGS-2 mRNA binding proteins), and/or changes in translational modulation. Therefore, to assess if transcriptional activation is involved in PGS-2 and *zif*-268 induced expression by kainic acid nuclear run-on, transcription assays were conducted. The pattern of transcriptional activation qualitatively matched the profile of changes in mRNA abundance (Fig. 2B). The level of relative stimulation of PGS-2 transcription was, however, less than the relative increases in mRNA. It should be noted that the methodologies used to obtain these two sets of values differ in several aspects and may not be readily comparable. As a consequence, *in vitro* transcription patterns are to be considered as analogous to mRNA abundance (18). Analogous, comparative patterns are also displayed in Figure 2A and B between *in vitro* transcription and mRNA abundance for *zif*-268. The transcriptional activation of PGS-1, as depicted in Fig 2B, displayed no induction up to 6 hours; however, there was a small increase after 72 hours of kainic acid treatment.

PGS-2 expression, as analyzed 2 hours after kainic acid injection, was highest in hippocampus (35-fold) followed by cerebral cortex (8-fold) (Fig. 1). While small increases were observed in brain stem and striatum, there were no changes in cerebellum. The largest induction of *zif*-268 was also observed in hippocampus (5.5-fold) followed by cerebral cortex (4.8-fold). Again, the changes observed in brain stem and striatum were small, with no detectable changes in cerebellum (Fig. 1). The intracellular PAF receptor antagonist BN50730 given intracerebroventricularly (icv) 15 minutes prior to kainic acid administration reduces both PGS-2 mRNA (data not shown) and protein (Fig. 3). Under the same conditions PGS-1 protein was not affected.

The platelet activating factor-prostaglandin G/H synthase-2 intracellular signaling pathway and apoptosis.

A PAF-stimulated signal transduction pathway is a major component of the kainic acid-induced PGS-2 expression in hippocampus. This conclusion is based upon the finding that a) PAF induces mouse PGS-2 promoter-driven luciferase activity in transfected cells, and BN 50730 inhibits this effect (6); and b) BN 50730 inhibits kainic acid-induced PGS-2 mRNA and protein accumulation in hippocampus by 90% (6). Both PAF (19) and PGS-2 (20) are potent mediators of the injury/inflammatory response. PAF (8,9) and PGS-2 (4,5) are also interrelated in neuronal plasticity. The PAF transcriptional activation of PGS-2 may provide clues about novel neuronal cell death pathways. The antagonist BN 50730 was much less effective against *zif*-268 expression. In fact, the delayed hippocampal induction of PGS-2 by kainic acid precedes selective neuronal apoptosis by this agonist in this neuroanatomical region (17).

In neurotrauma, cerebrovascular and neurodegenerative diseases, the significance of the PLA_2-related signaling triggered by ischemia reperfusion may be part of events finely balanced between neuroprotection and neuronal cell death. The precise events that would tilt this balance toward the latter are currently being investigated. We are exploring the hypothesis that PAF-COX-2 is a common signaling pathway involved in neurodegenerative diseases as diverse as retinitis pigmentosa and Alzheimer's Disease. It is interesting to note that PAF, being short-lived and rapidly degraded by PAF acetylhydrolase (10,21), behaves as a long-term signal with consequences to neurons through PGS-2 sustained expression. It is also interesting to highlight the fact that the PAF-PGS-2 pathway may be activated in neurons as an early event in the pathophysiology of several diseases, and although this pathway is a component of the "classical inflammatory" response, it is restricted to a disruption of intracellular signaling. In fact, apoptosis, by definition, lacks an initial inflammatory component. PGS-2 is localized in the nuclear envelope and perinuclear endoplasmic reticulum. The overexpression of hippocampal PGS-2 during cerebral ischemia and seizures may in turn lead to the formation of neurotoxic metabolites (e.g. superoxide). Current investigations aim to determine whether or not other messengers cooperate to enhance neuronal damage (e.g. nitric oxide) and the possible involvement of astrocytes and microglial cells. Further understanding of these potentially neurotoxic events involving lipid messengers and PGS-2 will permit the identification of new strategies and define therapeutic windows for the management of the inflammatory component in stroke, epileptic brain damage, head injury and neurodegenerative diseases.

Acknowledgments

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References

1. N.G. Bazan, E.B. Rodriguez de Turco, G. Allan, Mediators of injury in neurotrauma: Intracellular signal transduction and gene expression. J. Neurotrauma 12, 789-911 (1995).
2. E.A. Dennis, Diversity of group types, regulation and function of phospholipase A_2. J. Biol. Chem. 269, 13057-13060 (1994).
3. M. Kolko, M.A. DeCoster, E.B. Rodriguez de Turco, N.G., Synergy by secretory phospholipase A_2 and glutamate on inducing cell death and sustained arachidonic acid metabolic changes in primary cortical neuronal cultures. J. Biol. Chem. (in press).
4. K. Yamagata, K.I. Andreasson, W.E. Kaufmann, C.A. Barnes, P.F. Worley, Expression of a mitogen-inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids. Neuron 11, 371-386 (1993).
5. W.E. Kaufmann, P.F. Worley, J. Pegg, M. Bremer, P. Isakson, COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. Proc. Natl. Acad. Sci. 93, 2317-2321 (1996).
6. N.G. Bazan, B.S. Fletcher, H.R. Herschman, P.K. Mukherjee, Platelet-activating factor and retinoic acid synergistically activate the inducible prostaglandin synthase gene. Proc. Natl. Acad. Sci. 91, 5252-5256 (1994).
7. T. Shimizu and L. S. Wolfe. Arachidonic acid cascade and signal transduction. J. Neurochem. 55, 1-15, (1990).

8. G.D. Clark, L.T. Happel, C.F. Zorumski, Bazan NG, Enhancement of hippocampal excitatory synaptic transmission by platelet-activating factor. Neuron 9, 1211-1216 (1992).

9. K. Kato, G.D. Clark, N.G. Bazan, C.F. Zorumski, Platelet activating factor as a potential retrograde messenger in Ca[1] hippocampal long-term potentiation. Nature 367, 175-179 (1994).

10. N.G. Bazan, Inflammation: A signal terminator. Nature 374, 501-502 (1995).

11. V.L. Marcheselli, M. Rossowska, M.T. Domingo, P. Braquet, N.G. Bazan, Distinct platelet-activating factor binding sites in synaptic endings and in intracellular membranes of rat cerebral cortex. J. Biol. Chem. 265, 9140-9145 (1990).

12. S.P. Squinto, A.L. Block, P. Braquet, N.G. Bazan, Platelet-activating factor stimulates a Fos/Jun/AP-1 transcriptional signaling system in human neuroblastoma cells. J. Neurosci Res. 24, 558-566 (1989).

13. S.P. Squinto, A.L. Block, P. Braquet, N.G. Bazan, Platelet-activating factor activates HIV promoter in transfected SH-SY5Y neuroblastoma cells and MOLT-4 T lymphocytes. J. Mol Neurosci. 2, 79-84 (1990).

14. N.G. Bazan, S.P. Squinto, P. Braquet, T. Panetta, V.L. Marcheselli, Platelet-activating factor and polyunsaturated fatty acids in cerebral ischemia or convulsions: Intracellular PAF-binding sites and activation of a Fos/Jun/Ap-1 transcriptional signaling system. Lipids 26, 1236-1242 (1991).

15. J.L. Morgan and T. Curran. Proto-oncogene transcription factors and epilepsy. TIPS 12, 343-349 (1991a).

16. V.L. Marcheselli and N.G. Bazan. Platelet-Activating Factor Is a Messenger in the Electroconvulsive Shock-Induced Transcriptional Activation of c-*fos* and *zif*-268 in Hippocampus. J. Neurosci. Res.

17. H. Pollard, C. Charriaut-Marlangue, S. Cantagral, A. Repress, O. Robain, J. Moreau and Y. Ben-Ari. Kainate-induced apoptotic cell death in hippocampus. Neuroscience 63, 7-18 (1994).

18. V.L. Marcheselli, N.G. Bazan, Sustained induction of prostaglandin endoperoxide synthase-2 by seizures in hippocampus. Inhibition by a platelet-activating factor antagonist. J. Biol. Chem. 271, 24794-24799 (1996).

19. S.M. Prescott, G.A. Zimmerman, T.M. McIntyre, Platelet-activating factor. J. Biol. Chem. 265, 17381-17384 (1990).

20. N.G. Bazan, J. Botting, J.R. Vane (Eds.), New Targets in Inflammation: Inhibitors of COX-2 or Adhesion Molecules. William Harvey Press and Kluwer Academic Publishers, United Kingdom (1996).

21. Tjoelker, L.W., Eberhardt, C., Unger, J., Trong, H.L., Zimmerman, G.A., McIntyre, T.M., Stafforini, D.M., Prescott, S.M. and Gray, P.W. Plasma platelet-activating factor acetylhydrolase is a secreted phospholipase A_2 with a catalytic triad. J. Biol. Chem. 270, 25481-25487 (1995).

Figure Legends

FIG. 1. **Inhibition by BN 50730 of KA-induced PGS-2 mRNA (open bars) and *zif*-268 mRNA (closed bars) accumulation in rat hippocampus and cerebral cortex.** Animals were pretreated intracerebroventricularly with BN-50730 in DMSO (experimental) or DMSO alone (control) 15 min before KA injection. RNA was extracted for Northern blot analysis 2 hours after KA injection. (n=6 from 2 separate experiments, error bars ± 1 s. d. (Published with permission from ref. 18).

FIG. 2. **Time course of changes in relative mRNA abundance and transcriptional activity in hippocampus after KA treatment or a single electroconvulsive shock (ECS).**

(A) Relative abundance of PGS-2 and *zif*-268 mRNAs compared to GAPDH mRNA, as assessed by northern analysis. (n=9-12 for each time point from 3 separate experiments, error bars ± 1 s.d.)

(B) Transcriptional activity of PGS-2, PGS-1 and *zif*-268 genes assessed by nuclear run-on transcription. (n=3-4 from 3 separate experiments). Data are normalized to transcriptional activity of GAPDH. (Published with permission from ref. 18).

FIG. 3. **Inhibition by BN 50730 pretreatment of KA-induced PGS-2 protein accumulation (open bars), but not of endogenous PGS-1 (closed bars) in rat hippocampus.**

(A) Representative Western blots. BN 50730 or vehicle treatments were as for Fig. 4. Samples were collected 6 hours after KA treatment.

(B) Quantification of Western blot data expressed as a percent increase over control (vehicle-pretreated) values. (n=10-12 from 3 separate experiments, error bars ± 1 s.d.) (Published with permission from ref. 18).

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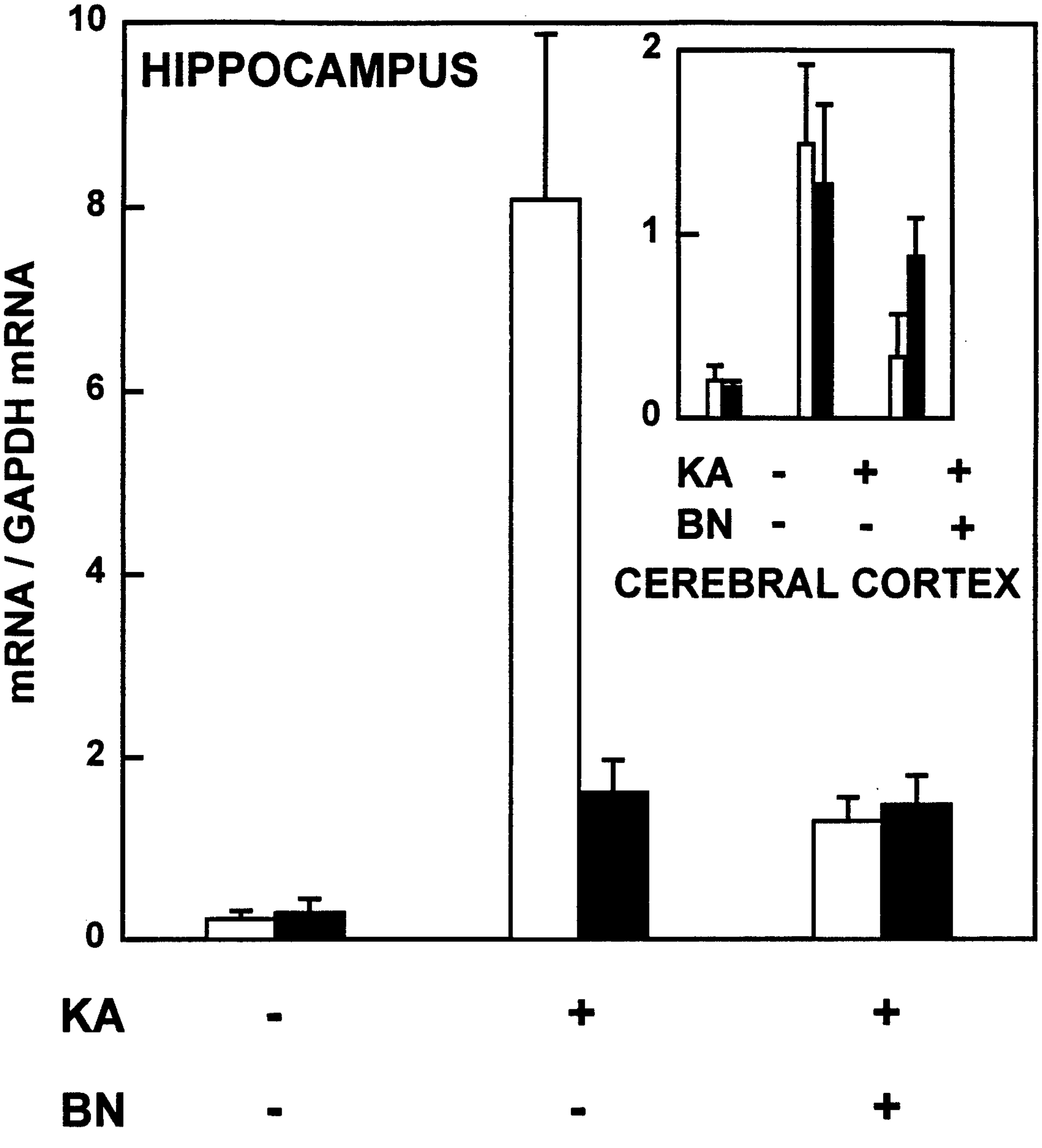
FIG. 4. **Excitatory synaptic transmission enhances the production of PAF, other messengers, and the expression of PGS-2.** NMDA-receptor activation by glutamate leads to phospholipase A_2 activation and the generation of PAF and arachidonic acid. PAF is synthesized through other metabolic routes as well (10). Other synaptic events, in addition to NMDA-receptor, may also be involved in the accumulation of PAF. PAF activates PGS-2 gene expression through a BN-50730-sensitive intracellular site, protein kinase cascades and transcription factors. The PGS-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). PGS-2 protein (COX-2) then catalyzes the conversion of arachidonic acid into PGH_2, the precursor of eicosanoids. Constitutive PGS-1 also catalyzes this metaboilc step (not shown). Prostaglandin E_2 is depicted as a product of PGS-2 (COX-2). The specific products of overexpressed PGS-2 are not yet known. PGE_2 is shown to potentially elicit genomic, paracrine and/or autocrine effects.


HIPPOCAMPUS
mRNA / GAPDH mRNA
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8
6
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KA - + +
BN - - +
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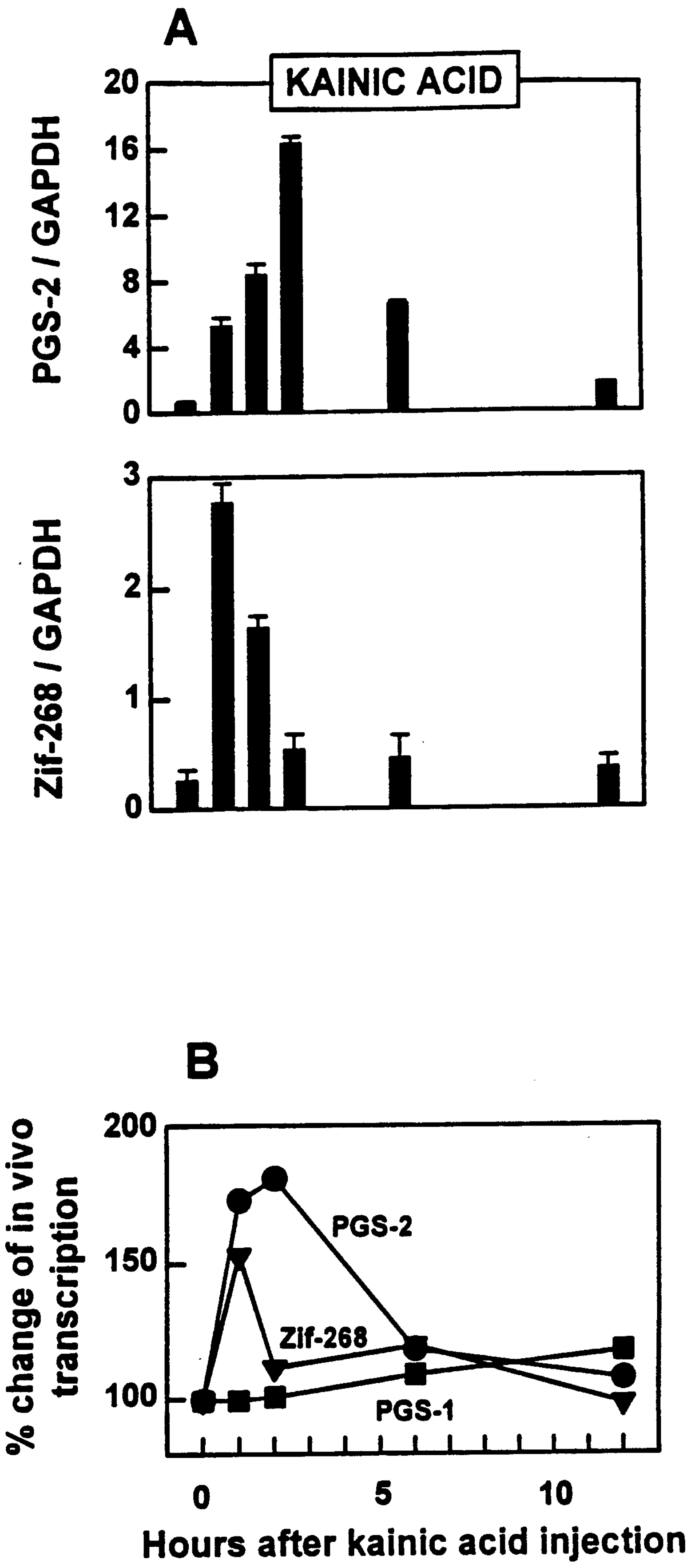
KA - + +
BN - - +
CEREBRAL CORTEX


A
KAINIC ACID
PGS-2 / GAPDH
0
4
8
12
16
20
Zif-268 / GAPDH
0
1
2
3
B
% change of in vivo transcription
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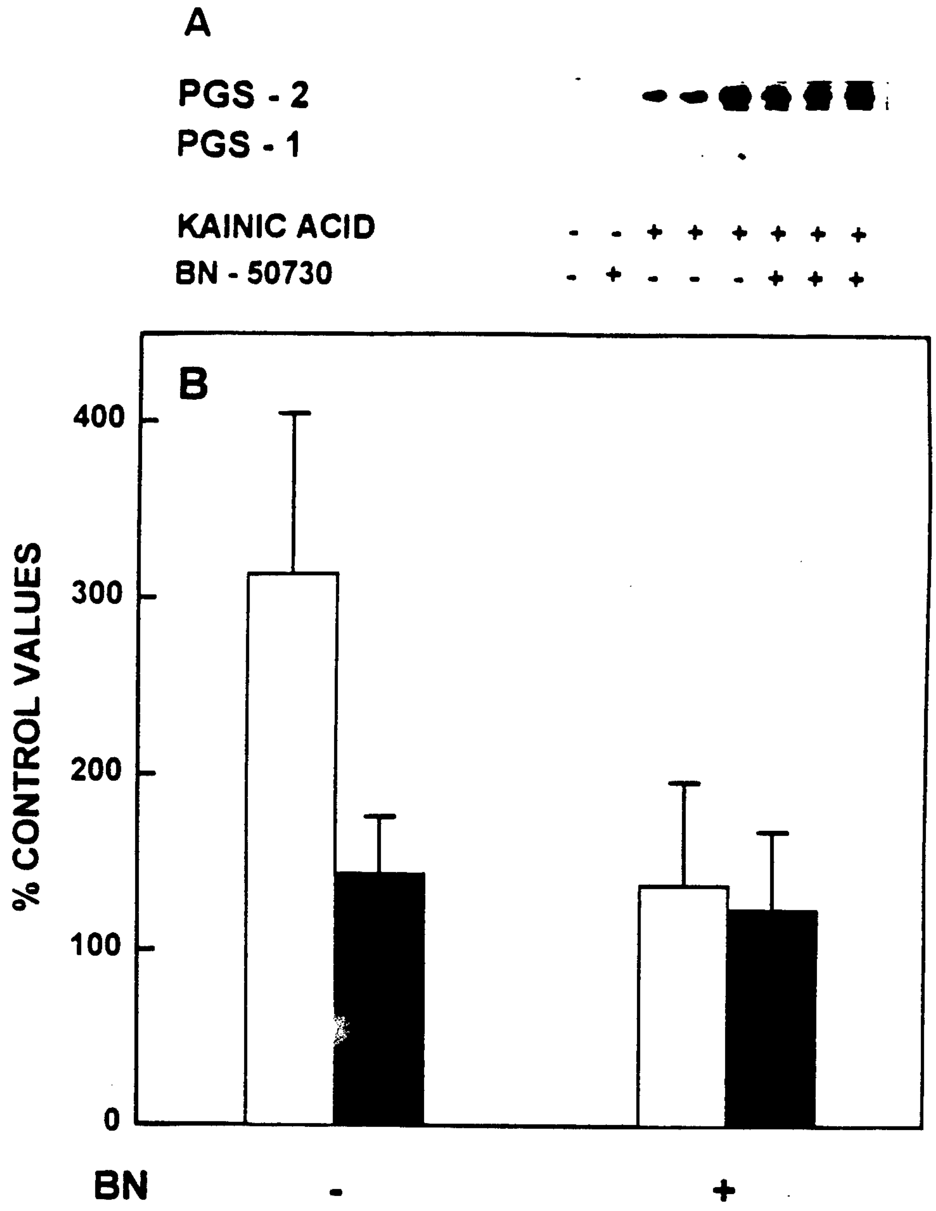
150
200
PGS-2
Zif-268
PGS-1
0
5
10
Hours after kainic acid injection


A
PGS - 2
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KAINIC ACID - - + + + + + +
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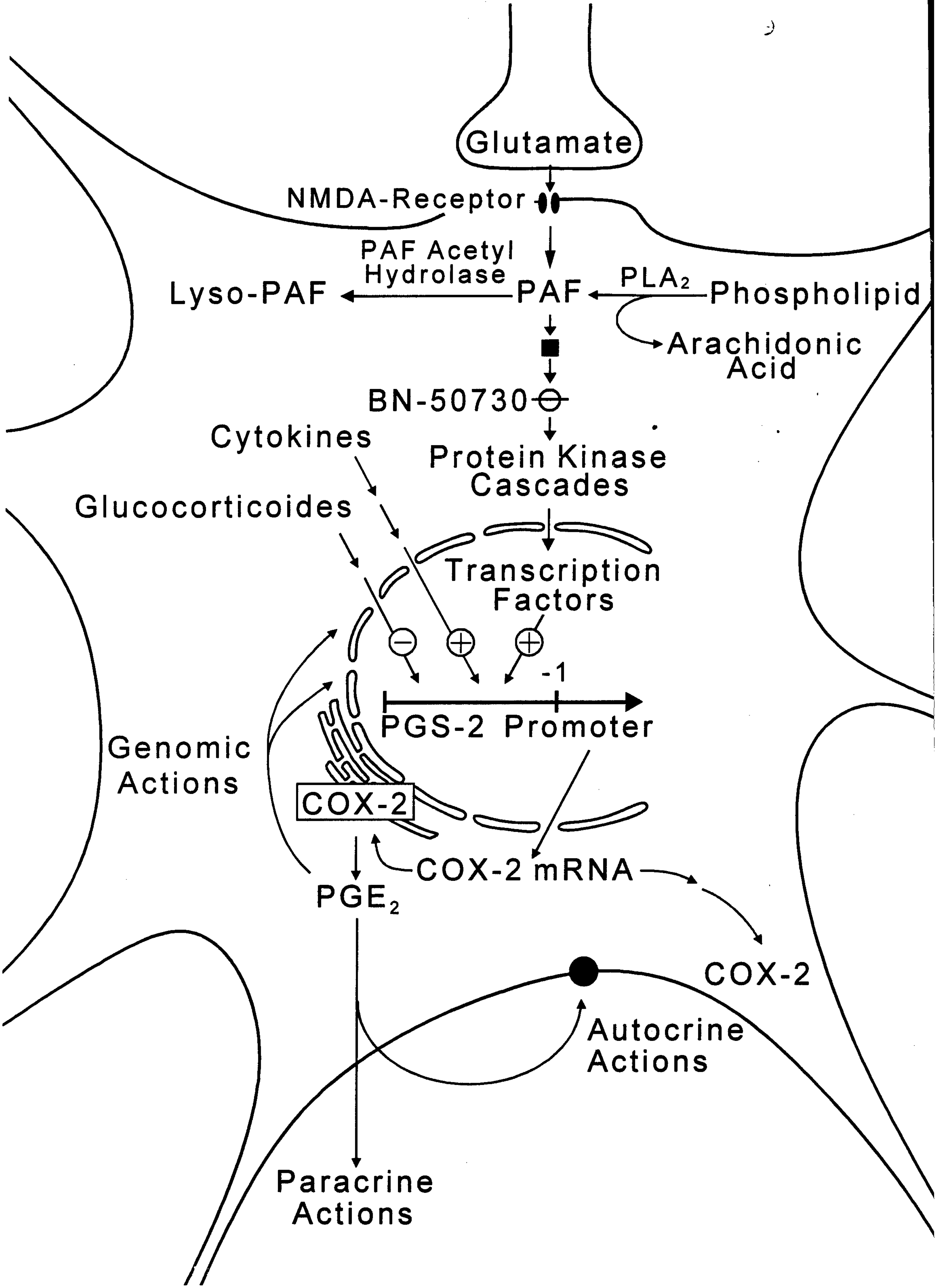
Glutamate
NMDA-Receptor
PAF Acetyl Hydrolase
Lyso-PAF
PAF
PLA₂
Phospholipid
Arachidonic Acid
BN-50730
Cytokines
Protein Kinase Cascades
Glucocorticoides
Transcription Factors
-1
PGS-2 Promoter
Genomic Actions
COX-2
COX-2 mRNA
PGE₂
COX-2
Autocrine Actions
Paracrine Actions

732.9

CALCIUM, GTP AND CARBACHOL STIMULATION OF HIPPOCAMPAL PHOSPHOINOSITIDE HYDROLYSIS FOLLOWING CHOLINERGIC DENERVATION AND SYMPATHETIC INGROWTH.
K.Kolasa*, D.Parsons and L.E.Harrell. Alzheimer's Disease Center, Dept.Neurology, VA&Univ.Alabama Med.Ctr., Birmingham, AL 35294.

Decreases in central cholinergic activity accompanied by increases in norepinephrine (NE) concentration have been described in Alzheimer's patients. To determine how these changes might affect brain biochemistry, our laboratory has utilized the model of hippocampal sympathetic ingrowth, in which peripheral sympathetic fibers, originating from the superior cervical ganglia, grow into the hippocampus following cholinergic denervation via medial septal lesions. Since sympathetic ingrowth can be prevented by ganglionectomy (Gx), the effects of cholinergic denervation alone (CD; MS lesions + Gx) can clearly be separated from the effects of hippocampal sympathetic ingrowth (HSI; MS lesion+sham Gx). HSI and CD have been found to differentially affect cholinergically stimulated phosphoinositide hydrolysis (PI) and the affinity of muscarinic receptors (mAChR) in a manner suggesting an alteration in coupling efficiency between the mAChR and PI turnover. To test this hypothesis, we have utilized newly developed methods in which prelabeled phosphoinositides (i.e., [^{3}H]-PI) are added to hippocampal membranes and hydrolysis assessed after the addition of calcium, to activate phospholipase C (PLC), GTPγS, to activate G-proteins, or GTPγS + carbachol, to activate the entire receptor complex. PLC activity in dorsal and ventral hippocampus was similar in HSI, CD and control animals. However, GTPγS stimulated PI hydrolysis in dorsal hippocampus was found to be significantly decreased in CD, when compared to control and HSI, animals ($p<0.02$), with HSI increasing PI turnover by 27% to control levels. Carbachol+GTPγS stimulated PI hydrolysis in dorsal hippocampus was also found to be decreased in CD when compared to control and HSI animals ($p<0.004$), with HSI increasing PI turnover by 15% to control levels. The results suggest that both sympathetic ingrowth and cholinergic denervation alone alter G-protein function, which may be the mechanism by which HSI and CD induce changes in mAChR affinity and cytosolic PI turnover.

732.10

SIGMA RECEPTOR REGULATION OF NMDA-STIMULATED [^{3}H]ARACHIDONIC ACID RELEASE FROM CEREBELLAR GRANULE CELLS IS PERTUSSIS TOXIN-SENSITIVE. G.M. Gonzalez-Alvear* and L.L. Werling. Dept. of Pharmacology, The George Washington University Medical Ctr., Washington, D.C. 20037.

We have previously reported that sigma (σ) receptors are involved in the regulation of arachidonic acid release from cerebellar granule cells. We have now investigated the effects of the sigma agonists (+)pentazocine and BD737 on NMDA-stimulated [^{3}H]arachidonic acid ([^{3}H]AA) release from cerebellar granule cells in the absence and presence of pertussis toxin.

Cerebella of eight-day old neonatal rats were dissected and chopped into pieces. Cells were mechanically and enzymatically dissociated, subjected to differential centrifugation, resuspended and plated on polyethyleneimine coated dishes. Cell cultures were treated with 10 μM cytosine arabinofuranoside to prohibit growth of non-neuronal cells. After at least eight days in culture, cells were incubated with 1 μCi/dish [^{3}H]AA for 24 hrs ± pertussis toxin (0.1 nM), then washed to remove residual, unaccumulated labeled eicosanoid. Release of [^{3}H]AA was stimulated by a 10 min exposure to 50 μM NMDA in the presence or absence of a sigma drug.

Increasing concentrations of sigma agonists (+)pentazocine and BD737 completely inhibited NMDA-stimulated [^{3}H]AA release from cerebellar granule cells. Furthermore, the sigma$_1$ antagonist DuP 734 reversed the inhibition of [^{3}H]AA release by both agonists. In contrast, (+)pentazocine and BD737 failed to inhibit [^{3}H]AA release from pertussis toxin-treated cells. These data suggest that sigma receptors regulating NMDA-stimulated arachidonic acid release in cerebellar granule cells are G_i or G_o protein coupled. (Supported by a grant from NIDA to LLW and by a NIGMS predoctoral fellowship to GMG.)

732.11

CHARACTERIZATION OF PLASMALOGEN-SELECTIVE PHOSPHOLIPASE A_2 FROM BOVINE BRAIN CYTOSOL. L.A. Horrocks*, H.-C. Yang and A. A. Farooqui, Dept. of Medical Biochemistry, The Ohio State University, 1645 Neil Ave., Rm. 465, Columbus, Ohio 43210.

The release of arachidonate from plasmalogen may be a receptor-mediated process catalyzed by 39 kDa plasmalogen-selective phospholipase A_2 (PLA_2). The purified enzyme is markedly inhibited by polyvalent anions such as phosphate, sulfate and citrate, with IC_{50} values of 400 mM, 150 mM and 5 mM, respectively, and stimulated by Triton X-100 and Tween-20. Octyl glucoside, sodium deoxycholate and taurocholate markedly inhibit enzymic activity. Non-specific inhibitors of PLA_2, such as quinacrine, nordihydroguaiaretic acid and manoalide, produce a dose-dependent inhibition. However, arachidonyl trifluoromethyl ketone (a potent inhibitor of 85 kDa cytosolic PLA_2) and bromoenol lactone (an irreversible inhibitor of 40 kDa plasmalogen-selective PLA_2 from myocardium) have no effect on enzymic activity. Finally, the 39 kDa plasmalogen-selective PLA_2 is inhibited by free fatty acids in a dose-dependent manner. Docosahexaenoate is the most potent inhibitor, followed by arachidonate, eicosapentaenoate and heptadecanoate. The inhibition by docosahexaenoate can be reversed with bovine serum albumin.

Supported by NIH grants NS-10165 and NS-29441.

732.12

INDUCIBLE PROSTAGLANDIN SYNTHASE AND *ZIF*-268 mRNA UPREGULATION IN VASOGENIC CEREBRAL EDEMA: INHIBITION BY A PAF ANTAGONIST. V.L. Marcheselli*and N.G. Bazan, Neuroscience Center, LSU Medical Center, New Orleans, LA 70112

Cerebral hypoxia, like global brain ischemia, triggers the release of glutamate which, in excess, leads to neuronal damage. Free fatty acids and diacylglycerols also accumulate *(BBA* **218**:1-10, 1970) due to activation of phospholipases A_2 and C. Platelet-activating factor (PAF), a product of PLA_2, is a mediator of neuronal damage. Prostaglandin H synthase (PGS, cyclooxygenase) is the rate-limiting enzyme in the synthesis of PGE_2, PGF_{2F}, PGD_2, prostacyclins, and thromboxanes. The inducible prostaglandin synthase mRNA (PGS-2, TIS-10), increases rapidly in response to a wide variety of stimuli such as mitogens and inflammation. Ischemia-reperfusion, single electroshock (ECS), and brain injury increase PGS-2 message levels in nervous tissue. After cryogenic injury, a model of vasogenic brain edema, the levels of PGS-2 mRNA rose rapidly reaching a peak at 2 hours, and remained upregulated after 24 hours. Levels of TIS-8 mRNA, a zinc-finger synthesis and a transcription factor, peaked after 1 hour of the injury and returned to control levels after 6 hours. The PAF antagonist BN-50730 partially inhibited the increase in both PGS-2 and TIS-8 mRNAs. Levels of tissue edema were measured by plasma Evans Blue extravasation. The animals pretreated with BN-50730 or dexamethasone were partially protected. Thus, PAF receptors may be involved in brain damage after injury and the PAF antagonists may be useful as pharmacological tools to protect the brain from that damage. Supported by DAMD-17-93-V-3013.

732.13

ACTIVATION OF THE PLA_2-AA SIGNALING CASCADE REGULATES 5-HT_{1A} RECEPTOR FUNCTION IN CHO CELLS. K.L. Jameson*[1], K.A. Berg[1], A. Saltzman[2] and W.P. Clarke[1]. Dept. of Pharmacology[1], Univ. Texas Health Science Center, San Antonio, Texas 78284 and Dept. of Molecular Biology[2], Rhone-Poulenc Rorer, Collegeville, PA 19426.

We have reported that CHO cells naturally express a 5-HT_{1B} receptor subtype (Berg *et al*, Mol Pharmacol. 1994. vol 46: 477- 484) which is regulated by a cyclooxygenase-dependent arachidonic acid (AA) metabolite produced when phospholipase A_2 (PLA_2) is activated with melittin or the purinergic receptor agonist ATP (Berg *et al*, *Soc. Neurosci.* 1994. vol 20:1158). Currently, we are investigating the effect of activation of the PLA_2-AA signaling cascade on the 5-HT_{1A} receptor system in CHO cells expressing the human 5-HT_{1A} (G21) receptor (≈ 130 fmol/mg protein). The inhibition of forskolin (1 μM)-stimulated cAMP accumulation (FScA) by dipropyl-5-carboxamidotryptamine (dp-5-CT) in the presence and absence of melittin (2.5 μg/ml) or ATP (1 mM) was measured. In control experiments, both melittin and ATP increased AA release by 620% and 100% above basal, respectively, and, as we found previously, reduced 5-HT_{1B}-mediated inhibition of FScA. Melittin also reduced 5-HT_{1A} responsiveness. The EC_{50} for dp-5-CT was shifted to the right ($pEC_{50} = 7.53 \pm 0.06$ {30 nM} vs 7.74 ± 0.05 {18 nM}; $p \leq 0.05$; n=4) and the maximal inhibition was reduced (74% ± 2% vs 83% ± 1%; $p \leq 0.01$) in the presence and absence of melittin, respectively. In contrast, ATP had no effect on 5-HT_{1A}-mediated inhibition of FScA (n=2). Thus, although the responsiveness of the 5-HT_{1A} receptor system may be regulated by the PLA_2 -AA signaling cascade, the mechanism of this regulation may differ from that for the 5-HT_{1B} receptor system. (Supported in part by USPHS grants HD 26437, and MH 48125)

732.14

SPREADING DEPRESSION ELEVATES INDUCIBLE CYCLOOXYGENASE IMMUNOREACTIVITY IN DISCRETE CORTICAL REGIONS. R.P. Kraig*, C. Breder & P. Park. Dept. Of Neurology, The University of Chicago, Chicago, IL

Eicosanoids are powerful paracrine signals which may be involved in such diverse phenomena as conduction of messages in sensory neuronal pathways and the transformation of glia into reactive species. Spreading depression (SD) is a phenomenon that can induce gliosis uncompromised by cellular necrosis. Accordingly, to begin examining how eicosanoids might influence gliosis we characterized how recurrent SD altered the immunoreactivity (ir) for the inducible form of cyclooxygenase (iCOX).

Male, Wistar rats (n=72) were anesthetized with halothane. SD was induced in parietal cortex by superfusion of potassium chloride (1.0M, 3 hrs). Micropipettes placed in both frontal cortices recorded the occurrence of SD ipsilateral to the stimulus and its absence in contralateral, control cortex. Animals were allowed to recover for 1, 3, & 6 hr; and 1, 2, 3, 7, 14, 21, & 28 days. They were then re-anesthetized and processed for iCOX-ir. Blocking studies established the specificity of immunostaining. Computer-based and semi-quantitative image analyses of the log ratio of left, experimental versus right, control cortex were done in six distinct cortical regions showing iCOX-ir: insular, motor, piriform, and perirhinal cortex as well as the lateral nucleus of the amygdala and hippocampus. SD caused a significant ("p" values varying between less than 10^{-6} to 0.05) increase in iCOX-ir from 3 hr to 3 days after SD in all brain regions except amygdala and hippocampus. The lack of significant change in these two zones may stem from a symmetric increase in iCOX-ir. Pretreatment with indomethacin and mepacrine had no affect on SD-induced iCOX-ir changes while pretreatment with dexamethasone and L-NAME reduced the rise in iCOX-ir to a nonsignificant difference between sides. Treatment with sodium nitroprusside and ephedrine also altered iCOX-ir.

These results show that neocortical SD results in a significant rise in iCOX-ir within discrete cortical regions. Furthermore, these rises in iCOX-ir can be reduced by modulation of eicosanoid and nitric oxide metabolism.

248.3

EXPRESSION OF GLUTAMATE RECEPTORS OF RAT CEREBELLAR GRANULE CELLS DEPENDS UPON THE VOLUME OF CULTURE MEDIUM.

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In a preliminary series of studies we have found that the response of cerebellar granule cells cultured in vitro for 8 days to a cytotoxic glutamate pulse (100uM, 20 min. incubation) varies according to the volume of medium in which neurons are grown. When cells are cultured in low volume (LV) ($1.2\text{x}10^6$ cells/1.2 ml/17 mm dishes) the glutamate pulse causes 80-90% cell death. When sister cultures are prepared in a high volume (HV) of 4.0 ml the cell death following glutamate treatement is reduced to 20-30%. Addition of a conditioned medium (CM) derived from LV cultures to HV cultures markedly increases their response to the toxic gluatamate treatement. In order to investigate this volume dependency and the action of CM on glutamate sensitivity, we have measured sodium currents, the currents evoked by AMPA/kainate and NMDA as well as the mRNA (1A, 1B, 2A,2B,2C) and the protein subunits forming the AMPA/Kainate (GluR2/3,GluR6/7) and NMDA (NMDAR1,2A/B) receptors. We found that the glutamate resistant phenotype ensuing in HV culture conditions is accompanied by, and probably causally connected with, a lowered functional and physical expression of both voltage operated sodium channels as well as kainate and NMDA receptors.

We hypothesize that such volume dependency to the cytotoxic action of glutamate is connected with the extent of production and release in culture of a substance operationally defined as glutamate sensitizing activity or GSA.

248.4

ANTISENSE OLIGONUCLEOTIDES TO CALPAIN I mRNA PROTECT CULTURED HIPPOCAMPAL SLICES FROM NMDA-INDUCED PATHOPHYSIOLOGY.

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Two measures of NMDA-induced excitotoxic damage were reduced in cultured hippocampal slices that were previously exposed to antisense oligonucleotides (25μg/ml for 5 days) directed against the mRNA for calpain I. First, antisense intervention attenuated the generation of spectrin breakdown products (BDP's) following a 10-20 min NMDA (200 μM) infusion by approximately 42% ($p<.04$, t-test, 2 tails) on immunoblots developed with a BDP-specific antibody. These reductions in BDP formation were associated with a comparable degree of protease suppression, as measured by decreases in immunoblots (~42%) and *in vitro* activity estimates (~60%, $p<.01$, t-test, 2 tails), strengthening the assumption that increases in the concentration of spectrin fragments indeed reflect recent episodes of calpain I activity. Second, translational suppression of calpain I protected CA1 neurons from enduring NMDA-induced (200 μM for 15 min) synaptic dysfunction. Although slices transfected with antisense probes mimicked sibling cultures treated with an equivalent concentration of sense oligonucleotides in their initial response to NMDA (complete loss of synaptic potentials), they recovered more quickly and to a greater extent (to 74% of baseline EPSP amplitude after 55 min, $p<.03$, t-test, 2 tails) than did sense controls (to 21% of baseline at same time point). These results suggest that proteolysis by calpain I plays a significant role in excitotoxicity and that translational suppression of the protease protects cultured slices from the extreme spectrin proteolysis and pathophysiology normally elicited by intense NMDA receptor activation (supported by NIA AG00538 and NIMH MH14599).

248.5

OVEREXPRESSION OF RECOMBINANT HUMAN CALPASTATIN IN *E. COLI* AND ITS NEUROPROTECTIVE EFFECTS AGAINST EXCITOTOXICITY

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Calpastatin is an endogenous inhibitor acting specifically on the calcium-activated neutral proteinases (calpains), which have been implicated in neurodegenerative processes including Alzheimer's disease. Two species of calpastatin with molecular weights of 110 (tissue-type) and 70 kDa (erythrocyte-type) have been described in mammals (Inomata et al., '93). The larger species of calpastatin consists of five domains L, 1, 2, 3 and 4 among which domains 1, 2, 3, and 4 are repetitive and share homology with each other. Calpain inhibitory activities have been observed for each of the repetitive domains. The function of the domain L is not known. To further our understanding of the structure-function relationship of calpastatin molecules and to explore neuroprotective effect of calpastatin, we overexpressed each domain or combination of domains of calpastatin in *E. coli*. Single and multiple domains of calpastatin were cloned by reverse transcriptase polymerase chain reaction (RT-PCR) using human neuroblastoma mRNA as templates or by PCR using human liver or brain cDNA as templates. Each of the domain(s) L, 1, 1-2, 1-3, and 4 was cloned in T7 polymerase driven *E. coli* expression vectors. The level of recombinant DNA expression was 10% or greater of the total protein for each construct. Recombinant calpastatins D1, D1-2, D1-3 and 4 showed high specific activity toward calpain and demonstrated neuroprotection at an equal or higher level achieved by an NMDA receptor antagonist MK801 against glutamate excitotoxicity in mouse primary cortical neurons in culture. This neuroprotection was abolished by preabsorbing recombinant calpastatin with calpastatin antibodies. The result strongly indicates that the recombinant calpastatin may be a potential therapeutic agent of neurodegenerative diseases. The mechanism of the neuroprotection by calpastatin is being investigated.

248.6

ROLE OF CALPAIN IN THE DEVELOPMENT AND RECOVERY OF EXCITOTOXIC DENDRITIC INJURY IN VITRO.

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One of the earliest morphological changes following NMDA receptor activation in cultured cortical neurons is appearance of focal swellings along dendrites. The formation of these dendritic varicosities is largely calcium dependent and may be reversible. Also, the microtubule-stabilizing compound, taxol, attenuates NMDA-induced dendritic varicosity formation in mouse cortical cell cultures (Soc. Neurosci. Abstr. 20:1529), suggesting that the microtubule cytoskeleton plays a role in the maintenance of dendritic structure. Because several cytoskeletal proteins are targets of the calcium-activated enzyme, calpain, we investigated the role of calpain in dendritic varicosity formation.

Application of 30 μM NMDA for 10 minutes caused widespread dendritic varicosity formation visualized by MAP2 immunofluorescence. Pre-treatment of neuronal cultures with 1-100 μM MDL28,170 (generously provided by Marion Merrell Dow Inc.) or Calpain Inhibitor I for 2 hours did not alter the formation of dendritic varicosities induced by NMDA. These calpain inhibitors did substantially attenuate the NMDA-induced loss of MAP2 and increase of spectrin BDP immunoreactivity, suggesting sufficient cell permeability of these compounds (see abstract by Meschia et al., this meeting).

The NMDA exposure used in these studies was sublethal, as assessed by LDH release measured 24 hours post-exposure, and dendrites returned to normal morphology within 2 hours following NMDA removal. However, the presence of calpain inhibitors pre- and post-treatment blocked this recovery for at least 8 hours. These results suggest that calpain activation does not play a role in varicosity formation, but may be necessary for recovery of dendritic structure following injury.

(Supported by NIH NS32140 and NS01543, to MPG)

248.7

NMDA INDUCES CALPAIN-MEDIATED PROTEOLYSIS OF MICROTUBULE-ASSOCIATED PROTEIN 2 AND SPECTRIN IN CULTURED NEURONS.

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Microtubule-associated protein 2 (MAP2) is a somatodendritic cytoskeletal element which is rapidly lost following cerebral ischemia or traumatic brain injury in vivo. The calcium-dependent neutral protease, calpain, may contribute to loss of cytoskeletal proteins and to neuronal injury in this setting. To assess the mechanisms and possible significance of calpain activation, we examined potential targets of calpain proteolysis in cortical cultures exposed to NMDA, using immunofluorescence and Western blotting.

Primary dissociated neurons from mouse neocortex were exposed to 30 μM NMDA for periods of 10 to 120 min. NMDA exposures of 20 min resulted in widespread neuronal death one day later. Immediately following NMDA exposure cultures were fixed and processed for indirect immunofluorescence, or were harvested and total MAP2 was measured using 7.5% SDS-PAGE followed by Western blotting and enzymatic chemiluminescence. A decrease in MAP2 immunoreactivity was first detectable with 60 min of continuous NMDA exposure, and MAP2 was virtually absent with 120 min exposure. There was little appreciable loss of MAP2 during 120 min NMDA exposure when calcium was omitted from the exposure medium. NMDA exposure (60-120 min) also resulted in appearance of calpain-specific spectrin breakdown products (Ab provided by R. Siman, Cephalon, Inc.). Addition of the calpain inhibitor, MDL28,170 (1-100 μM), substantially attenuated the loss of MAP2 immunoreactivity but did not reduce neuronal cell death. These observations suggest that sustained NMDA receptor stimulation in cortical neurons activates calpain, resulting in delayed proteolysis of MAP2 and spectrin. However, calpain activation may not be required to initiate irreversible neuronal death. (Supported by NIH NS32140 and NS01543, to MPG).

248.8

EFFECT OF SECRETORY PHOSPHOLIPASES A_2 AND GLUTAMATE ON VIABILITY OF RAT CORTICAL NEURONS AND CALCIUM DYNAMICS.

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Nonpancreatic secretory phospholipases A_2 ($sPLA_2s$) may be involved in modulation of neuronal function. A receptor binding $sPLA_2s$ has been cloned from muscle (Lambeau, et. al. JBC, 1994). Two $sPLA_2s$ from snake venom (OS_1 and OS_2) bind to this receptor. OS_2 and $sPLA_2$ from bee venom (BV) bind avidly to brain membranes, while OS_1 does not. We evaluated the neurotoxicity of OS_1, OS_2 and BV combined with glutamate (GLU). Lactate dehydrogenase (LDH) release was used as the toxicity assay. BV and OS_2 dose dependently (0.01-10 μg/mL) caused neurotoxicity and OS_1 did not. GLU (80 μM) was as toxic as approximately 0.5 μg/mL BV. Submaximally toxic concentrations of BV combined with GLU demonstrated higher toxicity levels than the two compounds added separately. BV (0.05 μg/mL) and GLU together showed three times more toxicity than the sum of the separate toxicity levels, indicating that GLU and BV are having a synergistic effect. The toxicity experiments were combined with studies investigating the effect of the $sPLA_2s$ on intracellular free calcium concentration ($[Ca^{2+}]_i$). The fluorescent calcium indicator fluo-3 was used with a confocal microscope to measure real-time calcium dynamics in these neurons. We observed basal oscillations in $[Ca^{2+}]_i$ in the cultures. BV and OS_2 dose-dependently (0.5-10 μg/mL) altered ($[Ca^{2+}]_i$) dynamics, while OS_1 had no effect. Both BV and OS_2 (0.5 - 10 μg/mL) obliterated calcium oscillations, and also decreased $[Ca^{2+}]_i$ to below baseline levels. We did not see any calcium modulations in the cultures exposed to $sPLA_2$ concentrations below 0.5 μg/mL, even though the $sPLA_2s$ were toxic to about 0.025 μg/mL. These results indicate that calcium-independent toxicity may be occurring at low $sPLA_2$ concentrations. Our results support the observed binding properties of $sPLA_2s$ in muscle and brain tissue, and provide evidence for modulatory roles of $sPLA_2s$ in neuronal signal transduction. (Supported by DAMD-17-93-V-3013).

444.1

INTERCELLULAR CALCIUM WAVES PROPAGATED VIA GAP JUNCTIONS IN NEURONS A.C. Charles* and R.F. Tyndale Dept. Of Neurology, UCLA, Los Angeles, CA 90024 and Addiction Research Foundation and Dept. Of Pharmacology, Univ. Of Toronto, Canada M5S 2S1

Spontaneous waves of increased intracellular calcium concentration were rapidly propagated over groups of primary mouse cortical neurons and immortalized hypothalamic (GT1-1) neurons in culture. Ca^{2+} waves were propagated at a rate of 100-200 μm/sec over 10-200 cells. Ca^{2+} waves were abolished by the removal of extracellular calcium and by TTX. Similar intercellular Ca^{2+} waves were induced by mechanical stimulation of a single cell. GT1-1 neurons showed flourescence recovery after photobleaching of a single cell, and intercellular Ca^{2+} waves were abolished by the gap junction blocker octanol. By contrast, a different clone of the GT1 neurons (GT1-7) showed frequent spontaneous Ca^{2+} oscillations but no intercellular Ca^{2+} waves, no intercellular communication of the response to mechanical stimulation, and no fluorescence recovery after photobleaching. Comparison of expression of connexin mRNA in the GT1-1 and GT1-7 lines using RT-PCR revealed a 5-fold greater level of connexin26 mRNA in the GT1-1 line, but no difference in the levels of connexin32 or connexin43 mRNA. These results show that neurons are capable of extensive Ca^{2+} signaling via gap junctions, and suggest that connexin26 is the gap junction protein which enables intercellular Ca^{2+} signaling in GT1 neurons. Intercellular Ca^{2+} waves in cultured neurons may represent a model for gap-junctional signaling between neurons in the developing nervous system, and between subsets of neurons in the adult brain.

444.3

ASSOCIATION OF TYPE I AND TYPE III INOSITOL 1,4,5 TRISPHOSPHATE RECEPTORS.

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The Inositol 1,4,5-trisphosphate receptor (IP_3R) is an intracellular calcium channel involved in coupling cell membrane receptors to calcium signal transduction pathways within the cell. The IP_3R is believed to form a tetrameric structure to produce the calcium channel in endoplasmic reticulum membranes. Several isoforms (I, II, III) of IP_3Rs have been identified which are coded by separate genes, and are expressed in many tissues with differing patterns of cellular expression. We have generated specific affinity purified polyclonal anti-peptide antibodies to each of the three isoforms. Western Blot analysis of RINm5F and ATt20 cells shows high levels of endogenously expressed type I and type III IP_3R, but undetectable levels of type II. Co-immunoprecipitation experiments were performed by immunoprecipitating from these cells with the type I specific antibody and Western Blotting with the type III specific antibody, or by immunoprecipitating with the type III specific antibody and Western Blotting with the type I specific antibody. Both experiments yielded a band at 260 kDa, the appropriate size of both the type I and type III IP_3R. Immunocytochemistry performed on these cell lines with either antibody demonstrated similar ER staining patterns. The type III IP_3R was absent from the secretory granules of ATt20 cells. These data indicate that type I and type III IP_3Rs can associate into a molecular complex.

444.5

CLONING AND SEQUENCING OF AN IP_3-RECEPTOR cDNA FROM LOBSTER OLFACTORY ORGAN. S.D. Munger *[1,2], B.W. Ache[1,2,3] and R.M. Greenberg[1]. Whitney Laboratory[1] and Depts. of Neuroscience[2] and Zoology[3], Univ. of Florida, St. Augustine, FL 32086.

Several lines of evidence suggest that inositol 1,4,5-trisphosphate (IP_3)-receptors (IP_3Rs) occur in the plasma membrane of neuronal and nonneuronal cells, but little is known about the structural similarities of these plasma membrane IP_3Rs to the better known intracellular IP_3Rs. IP_3 directly gates two types of ion channels in the plasma membrane of lobster olfactory receptor neurons (ORNs); these channels are functionally similar to IP_3Rs localized to endoplasmic reticulum (ER) and nuclear membranes in vertebrates (Fadool & Ache, *Neuron*, 9: 907; Hatt & Ache, *PNAS*, 91: 6264). A polyclonal antibody directed against the ER IP_3R of rat cerebellum recognizes membrane proteins of appropriate size in lobster ORNs; this antibody also perturbs function of the lobster IP_3Rs in excised patch recordings (Fadool & Ache, *ibid.*). We have exploited the suggested structural similarities between plasma membrane and ER IP_3Rs by amplifying a partial cDNA, homologous to known IP_3Rs, from reverse transcribed lobster olfactory organ RNA using degenerate primers and PCR. We extended the clone to the 3'-noncoding region using 3'-RACE (Rapid Amplification of cDNA Ends). We have constructed an IP_3R mini-cDNA library, and have isolated overlapping clones by plaque hybridization and PCR. The open reading frame of the cDNA isolated to date, which codes for 1400 amino acids and comprises *ca.* 50% of the anticipated coding region, exhibits 40-45% identity to known IP_3Rs. While Northern analysis demonstrates a low level of expression of a >10 kb message in the brain, but none in the nose, the more sensitive ribonuclease protection assay shows the message to be expressed in the nose and, at higher levels, in the brain. We are currently working to extend the cDNA to the 5'-end and to localize the receptor by *in situ* hybridization and immunohistochemistry. {This work supported by ONR grant N00014-90-J-1566}

444.2

THYROID HORMONE CONTROL OF CALCIUM WAVES IN *XENOPUS LAEVIS* OOCYTES. L. M. John, J. D. Lechleiter, and P. Camacho*. Dep't of Neuroscience, Univ. of Virginia, Charlottesville, VA 22903.

Inositol 1,4,5 trisphosphate (IP_3)-induced intracellular Ca^{2+} release mediates the action of many neurotransmitter signalling pathways. Spiral Ca^{2+} wave propagation and annihilation of IP_3-induced Ca^{2+} release reveals an underlying excitable process which can be accounted for by the Ca^{2+} dependent properties of the IP_3-bound IP_3 receptor (IP_3R). We previously demonstrated the importance of cytosolic Ca^{2+} buffering on this dynamic process by overexpression of sarco-endoplasmic reticulum Ca^{2+}-ATPases (SERCAs) in *Xenopus* oocytes (Science **260**, 226-229). Thyroid hormones and growth hormones have been shown to increase the expression of Ca^{2+}-ATPases in smooth muscles and cardiac myocytes. Additionally, thyroid hormones have been implicated in the regulation of Ca^{2+} uptake and/or release in mitochondria. Given the importance of Ca^{2+} sequestration in the dynamics of Ca^{2+} wave activity, we examined the effects of thyroid hormones on IP_3-induced Ca^{2+} signalling in *Xenopus* oocytes. To accomplish this, oocytes were injected with mRNA encoding the *Xenopus* thyroid receptor (TRβA1) and assayed for IP_3-induced Ca^{2+} wave activity 1-3 days later using confocal imaging. Exposure of oocytes expressing TRβA1s to L-3,3',5-triiodothyronine (T_3, 50-100 μM for 20-60 min before recording) resulted in an increase in the number of oocytes exhibiting IP_3-induced regenerative Ca^{2+} wave activity (58%; n=65) compared with control, non-mRNA injected oocytes (21%; n=24). In addition, TRβA1 expressing oocytes showed an increase in Ca^{2+} wave amplitude from 0.77±0.49 (ΔF/F) to 0.95±0.41and an increase in interwave periods from 24.4±11.3 to 64.9±42s in the presence of T_3. We conclude that acute exposure to thyroid hormone dynamically modulates IP_3-mediating Ca^{2+} signalling. These data will be discussed in relation to the action of thyroid hormone on mitochondrial Ca^{2+} buffering. The work was supported by NIH GM48451 and AHA878.

444.4

CONFOCAL MEASUREMENTS OF BASELINE NUCLEAR AND CYTOPLASMIC FLUORESCENCE: COMPARISON OF CA^{2+} INDICATOR AND NON-CA^{2+} INDICATOR DYES. M.N. Rand*, S. Agulian, & J.D. Kocsis, Dept. of Neurology and Sect. Neurobiology, Yale Medical School, New Haven, CT 06510; and VAMC, West Haven, CT 06516.

At baseline resting potentials, neurons which have been loaded with Ca^{2+} indicator dyes using a micropipette have higher levels of fluorescence in the nucleus than in the cytoplasm, and it has been suggested that this effect is due to the presence of more dye in the nucleus. To evaluate this idea confocal microscopy was used to compare nuclear to cytoplasmic fluorescence ratios (N/C ratios) of cultured adult rat dorsal root ganglion neurons filled by micropipette with either Ca^{2+} indicator or non-Ca^{2+} indicator dyes. Both 10 kD dextran-conjugated and free forms of the Ca^{2+} indicator and non-Ca^{2+} indicator dyes were used. In all cases, N/C ratios of Ca^{2+} indicator dyes were significantly higher than those of the non-Ca^{2+} indicator dyes (dextran-conjugated: 1.89 vs 1.11; free dye: 3.30 vs 1.58). N/C ratios of the non-Ca^{2+} indicator dyes remained constant whereas N/C ratios of Ca^{2+} indicator dyes varied significantly over time. Some of the neurons also developed blebs on their plasma membrane which filled with water, aqueous solutes and dye; for non-Ca^{2+} indicator dyes bleb fluorescence was always higher than nuclear fluorescence, and for Ca^{2+} indicator dyes nuclear fluorescence was always higher than bleb fluorescence. These results suggest that micropipette dye-loaded neurons have higher levels of nuclear Ca^{2+} at baseline resting potentials, and have important implications for the evaluation of depolarization-induced intracellular Ca^{2+} signals. Supported in part by the NIH and Department of Veterans Affairs.

444.6

PLATELET-ACTIVATING FACTOR INDUCED INTRACELLULAR CALCIUM OSCILLATIONS IN RAT HIPPOCAMPAL NEURONS. M. A. DeCoster, H. E. P. Bazan*, and N. G. Bazan. LSU Medical Center, Neuroscience Center, New Orleans, LA 70112-2234

As has been previously shown, we have found using confocal microscopy and fluorescent calcium indicators, that intracellular calcium concentration ($[Ca^{2+}]_i$) oscillates spontaneously in rat hippocampal neurons *in vitro*. While addition of glutamate (GLU) to these hippocampal cultures causes distinct $[Ca^{2+}]_i$ changes ranging from transient, single spikes (100-500 nM GLU) to sustained increases (20-80 μM GLU), GLU does not appear to induce $[Ca^{2+}]_i$ oscillations. We have investigated the ability of the potent lipid mediator platelet activating factor (PAF) to affect $[Ca^{2+}]_i$ dynamics in hippocampal neurons. When 4 μM methylcarbamyl PAF (mcPAF) was added to the hippocampal neurons, the average $[Ca^{2+}]_i$ was increased slightly in cells. Furthermore, the variance of fluorescence values after mcPAF additions was 8-fold higher than before additions, indicating an increase in oscillatory $[Ca^{2+}]_i$ dynamics induced by PAF. Neurons not spontaneously oscillating were observed to be induced to oscillate by PAF addition, and neurons spontaneously oscillating increased in oscillatory behavior upon PAF addition. In agreement with Bito *et al.* (Neuron, 9:285, 1992) we found that not all neurons responded to acute PAF application. In contrast, long-term effects of PAF treatment on hippocampal cultures appeared to affect the majority of cells. Overnight treatment with PAF (200-400 nM) and mcPAF (2-4 μM) reduced the neuronal $[Ca^{2+}]_i$ changes induced by GLU the next day when compared with cells pretreated with lysoPAF (2-4 μM) or the vehicle alone. In two cases, the $[Ca^{2+}]_i$ increases in response to 500 nM GLU were completely inhibited by PAF pretreatment. Since PAF has been shown to enhance hippocampal excitatory synaptic transmission (Clark et al., Neuron 9:1211, 1992) we postulate that induction of $[Ca^{2+}]_i$ oscillations by PAF may be an early signal of GLU release, resulting in GLU receptor desensitization (Supported by DAMD-17-93-V-3013).

PAF MODIFIES CELL FUNCTION AND PATHOLOGY BY AFFECTING GENE EXPRESSION

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PAF, a potent bioactive phospholipid, influences gene expression, exocytosis of excitatory neurotransmitters, and other cellular functions through selective signal transduction pathways. PAF modulates neuronal plasticity such as long-term potentiation (*Neuron* **9**:1211-16, 1992; *Nature* **364**:175-9, 1994) and memory formation (*PNAS* **92**:5047-51, 1995). PAF is also a transcriptional activator of the expression of primary response genes that encode transcription factors (c-*fos*, c-*jun*, *zif*-268) and enzymes (COX-2, urokinase-plasminogen activator). The intracellular pathways leading to PAF-induced gene expression include MAP-kinase and other phosphorylation events. The pathological significance of PAF-induced COX-2 transcriptional activation was studied during neural cell damage in animals undergoing repeated seizures and vasogenic edema. Under these conditions PAF-mediated gene induction promotes prostaglandin synthesis and activates cell injury, setting in motion abnormal neural plasticity responses. Similar effects of PAF were found in epithelial cells. Therefore the PAF-induced COX-2 expression represents a widespread pathway linking mediators of inflammation through the genomic arm of signal transduction. One additional action of PAF at the gene level indicates the potential of being a central player in the ability of cells to manipulate their local environment through extracellular matrix remodeling. PAF activates the expression of matrix metalloproteinases-1 and -9 (*PNAS* **90**:868-82, 1993; *IOVS* **36**:345-54, 1995) and of urokinase-PA (*IOVS* Suppl. **35**:1520, 1994). The cell's ability to tightly regulate PAF synthesis and degradation, as evidenced by multi and complex degradative enzymes, tells us that this bioactive lipid has important functions, and also has pathophysiological significance in several diseases. PAF-mediated gene expression is a central event in several of these PAF activities. Supported by NIH grant NS23003 and DAMD17-93-V-3013.

SUNDAY PM FATTY ACIDS AND THEIR METABOLITES (L22-L25)

L22

INTRACELLULAR LOCALIZATION OF 5-LIPOXYGENASE: SURPRISING IMPLICATIONS FOR THE CELL BIOLOGY OF EICOSANOID SYNTHESIS AND ACTIONS. M. Peters-Golden. Univ. of Michigan Sch. of Med., Ann Arbor, MI 48109.

The enzyme 5-lipoxygenase (5-LO) initiates the conversion of arachidonic acid to leukotrienes (LTs), pro-inflammatory lipid mediators which play important roles in both normal host defense and in the pathogenesis of inflammatory disease states. Activation of 5-LO involves its Ca^{2+}-dependent redistribution from a soluble cellular compartment to a particulate compartment. It was initially presumed that the soluble and particulate sites of 5-LO localization in resting and stimulated cells represented the cytosol and plasma membrane, respectively. However, careful analysis using a combination of immunomicroscopic and biochemical techniques have revealed a number of surprising findings. First, in all cells examined thus far, the nuclear envelope represents the site to which 5-LO translocates upon agonist activation. Interestingly, the nuclear envelope is also the site at which a number of other proteins involved in LT synthesis are localized: these include cytosolic phospholipase A$_2$, 5-LO activating protein, and LTC$_4$ synthase. Second, in resting cells 5-LO may reside in the cytosol, within the euchromatin region of the nucleus, or in both of these compartments, depending on the cell type. Third, both cytosolic and nuclear pools of the enzyme are catalytically active and capable of translocating to the nuclear envelope upon agonist activation. However, these distinct pools of 5-LO exhibit different proclivities to translocate and do so with different kinetics. Fourth, the localization of 5-LO can be modulated by culture conditions as well as by cellular recruitment to sites of inflammation in vivo. The functional implications of 5-LO compartmentalization are as yet unclear. However, the unexpected complexity of, and significance of the nucleus in, the cell biology of 5-LO activation suggest potential novel functions of LTs and of the 5-LO protein itself.

L23

PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR α INHIBITS HEPATIC S14 GENE TRANSCRIPTION.

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The peroxisome proliferator-activated receptor (PPAR) has been implicated in fatty acid regulation of gene transcription. Lipogenic gene transcription is inhibited by polyunsaturated fatty acids (PUFA). We used the PUFA-sensitive rat liver S14 gene to examine the role PPAR played in fatty acid regulation of hepatic lipogenic gene transcription. Transfection of hepatocytes with S14CAT fusion genes showed that the negative PUFA-response elements (nPUFA-RE) were located within the proximal promoter region (-220/-80 bp), while the negative peroxisome proliferator response elements (nPPRE) were located to a region (-2.9/-2.5 kb) containing 3 thyroid hormone response elements (TREs). The S14TRE region was sufficient to confer negative control to a heterologous promoter following WY14,643/PPARα treatment. Gel shift analysis showed that PPARα did not bind the S14TRE region directly. However, functional studies showed that co-transfection of RXRα, but not the T$_3$ receptor-β1 (TRβ1), abrogated the inhibitory effect of PPARα on S14 gene transcription. We suggest PPARα inhibits S14 gene transcription by sequestering RXR, a factor required for TRβ1 action at the S14TRE. Since the cis-regulatory elements for PPARα and PUFA are spatially distinct, PPARα cannot be the mediator of PUFA-mediated suppression of S14 gene transcription. Supported by NIH DK43220.

L24

ISOPROSTANES: UNIQUE BIOACTIVE PRODUCTS OF LIPID PEROXIDATION. L. J. Roberts, II. Vanderbilt University, Nashville, Tn 37232.

Isoprostanes (IsoP's) are a series of prostaglandin(PG)-like compounds that are formed independent of the cyclooxygenase by free radical catalyzed peroxidation of arachidonic acid esterified to phosophlipids. New insights regarding the enzymatic hydrolysis of preformed esterified Isop's will be presented. The initial series of Isop's identified had a prostanoid F-type ring structure (F$_2$-Isop's). Subsequently, Isop's with prostanoid E-type and D-type ring structures were identified (E$_2$/D$_2$-Isop's). Additional unique products of this pathway (isothromboxanes and isolevuglandins) have been recently identified and will be discussed. Two Isop's that have been tested for bioactivity (8-iso-PGF$_{2\alpha}$ and 8-iso-PGE$_2$) have been found to be potent vasoconstrictors via interaction with a receptor(s). Isolevuglandins, on the other hand, exert unique biological properties owing to their remarkable proclivity to form covalent adducts with proteins and DNA. A valuable aspect of the discovery of Isop's is that measurements of Isop's in biological fluids and tissues offers a reliable means to assess oxidative stress status *in vivo*. Examples of how measurement of Isop's has proven useful to assess the role of free radicals in human disease, with an emphasis on atherosclerosis/cardiovascular disease, will be discussed.

L25

LIPID SECOND MESSENGERS AND THE UPREGULATION OF INDUCIBLE PROSTAGLANDIN SYNTHASE-2 TRIGGERED BY VASOGENIC CEREBRAL EDEMA.

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Cerebral hypoxia, like global brain ischemia, triggers the release of glutamate which, in excess, leads to neuronal damage. Free fatty acids and diacylglycerols also accumulate (Biochem. Biophys. Acta. 218, 1- 10, 1970) due to activation of phospholipases A2 and C. Platelet-activating factor (PAF), a product of phospholipase A2, is mediator of neuronal damage. Prostaglandin H synthase (cyclooxygenase) is a rate-limiting enzyme in the synthesis of *PGE*2, *PGF*2*F*, *PGD*2, prostacyclins, and thromboxanes. The inducible prostaglandin synthase (PGHS-2), and TIS-10, mRNA increases rapidly in single electroshock (ECS), and in ischemia reperfusion in brain. After cryogenic injury, a model of vasogenic brain edema, PGHS-2 mRNA rose rapidly reaching peak levels after 2 hours, and remained upregulated up to 24 hours. TIS-8 mRNA, a Zinc-finger synthesis protein and a transcription factor, peaked after 1 hour of the injury, and returned to control levels after 6 hours. The protein levels of PGHS-2 in brain cortex were elevated up to 23 times the control levels 6 hours after injury. In hippocampus PGHS-2 peak at five hours with three orders increase. The PAF antagonist BN-50730 partially inhibited the increase in both PGHS-2 and TIS-8 mRNAs. Edema levels were measured by plasma Evans Blue extravasation. The animals pretreated with BN-50730 or dexamethasone were partially protected. Bioactive lipid messengers like PAF and PAF receptors may be involved in brain damage after injury. Supported by DAMD-17-93-V-3013.

SATURDAY/SUNDAY PM LIPID SECOND MESSENGERS (L26-L27)

L26

Defects in sphingolipid synthesis render *S. cerevisiae* cells sensitive to Ca^{2+}. T. Beeler, D.Fu, E. Monaghan, K. Gable and T. Dunn Uniformed Services Univ. Health. Sci. Bethesda, MD 20814

Analysis of suppressors of two Ca^{2+}-sensitive mutants, *csg1* and *csg2*, suggested a relationship between the Ca^{2+}-sensitive growth phenotype of the *csg* mutants and sphingolipid metabolism. Mannosylation of inositolphosphoceramide (IPC) is defective in the *csg* mutants causing inhibition of MIPC and M(IP)$_2$C synthesis. Decreased accumulation of the sphingolipid species IPC-C decreases the Ca^{2+}-sensitivity of the *csg* mutants. Reduced accumulation of IPC-C results from mutations in serine palmitoyltransferase (Scs1p), treatment with an inhibitor of serine palmitoyltransferase, mutations in a hydroxylase (Scs7p) required for IPC-C synthesis, and increased expression of a copper transporter (Ccc2p) or increased levels of Cu^{2+} in the growth media which increases conversion of IPC-C to a hydroxylated IPC-D species. A working hypothesis is that Csg1p and Csg2p function to flip IPC-C across the ER or Golgi membrane where it is mannosylated; accumulation of IPC-C on the cytosolic side of the membrane leads to Ca^{2+}-induced death.

L27

Relationship between arachidonate-phospholipid remodeling and cell proliferation Marc E. Surette[*], James D. Winkler[‡] and Floyd H. Chilton[*§] Depts of [*]Medicine and [§]Biochemistry, Bowman Gray School of Medicine, Winston-Salem, NC 27157. [‡]Dept. Inflam. Pharmacol. SmithKline Beecham Pharm., King of Prussia, PA 19406.

Previous studies have demonstrated that the anti-proliferative agent ET-18-O-CH$_3$ as well as the structurally unrelated compounds SK&F 45905 and SK&F 98625 block CoA-independent transacylase (CoA-IT) activity. These compounds also dose-dependently induce apoptosis in HL-60 cells. Structural isomers of SK&F 45905 and SK&F 98625 which lack CoA-IT inhibitory activity fail to induce apoptosis. All three compounds induced a dose-dependent decrease in the mole quantity of AA associated with PE while increasing the AA content in both phosphatidylcholine (PC) and neutral lipids. The effect was specific to AA as no loss of either linoleic acid nor oleic acid from PE was observed. In addition to changes in phospholipids, CoA-IT inhibitors caused a marked increase in free AA levels and AA-associated with triglycerides. These data raised the question of whether free AA has a role in regulating cell proliferation and apoptosis. To test this hypothesis Triacin C, a compound that blocks fatty acid reacylation by inhibiting acyl-CoA synthetase, or free AA were provided to HL-60 cells. Both Triacin C and AA blocked thymidine incorporation in HL-60 cells after 24 hours. Taken together, these data suggest that compounds which uncouple reacylation leading to increases in free AA levels affect HL-60 cell proliferation. (Supported By NIH Grant AI24985 and by a Medical Research Council of Canada Centennial Fellowship)

1645

ALTERING NEUROTRANSMITTER LEVELS *IN VITRO* FOLLOWING GLUTAMATE DECARBOXYLASE GENE TRANSFER. J. D. Fritz, N. Hale, A. Utz, J. Northington, L. Wu, A. C. Powers, T. Verdoorn, and D. Robertson. Vanderbilt Univ., Nashville, TN 37232.

Adenoviral vectors encoding either isoform of human glutamate decarboxylase (GAD$_{65}$ or GAD$_{67}$) under the control of the CMV promoter were constructed. Expression of GAD$_{65}$ or GAD$_{67}$ was verified by Western blot analysis of rat pituitary cells 48 hours after adenoviral-mediated GAD (AdGAD) gene transfer. Transgene expression was observed in ~100% of rat cerebellar granule neurons 3 days after adenoviral-mediated β-galactosidase gene transfer. Extracellular [GABA] increased >25 fold (79 μM verses 3 μM) in neurons 9 days after AdGAD$_{65}$ gene transfer. Intracellular [GABA] also increased in these neurons (20 μM verses 0.0 μM) and remained as such from 3-9 days after vector addition. Intracellular glutamate concentration decreased almost 50% in neurons 3 days after AdGAD$_{65}$ gene transfer, but returned to baseline (50-60 μM) by 9 days after vector addition. Similar [GABA] and [glutamate] were observed in neurons following AdGAD$_{67}$ gene transfer and in non-neuronal cell lines. [GABA] and [glutamate] were determined by HPLC using a Pico-Tag column (Waters) for free amino acid analysis. These data suggest that glutamatergic neurons can produce and release GABA following AdGAD gene transfer *in vitro*. Conversion of glutamatergic neurons to those expressing both glutamate and GABA could yield therapies in a variety of neurodegenerative disorders.

1646

MODULATION OF CHRONIC NEUROTOXICITY BY COMBINED KAINATE AND GLUTAMATE TREATMENT OF HIPPOCAMPAL NEURONS. H. Cinar, M.A. DeCoster, and N.G. Bazan . LSU Medical Center, Neuroscience Center, New Orleans, LA 70112-2234.

Acute treatment with excitatory amino acids, (EAAs) such as kainate (KA) and glutamate (G) cause neurotoxicity characterized by rapid increases in second messengers such as intracellular calcium; however, cell death in this model is delayed until approximately 16-24 hours. While these mechanisms of EAA neurotoxicity are understood for acute treatment, less is known of the effects of chronic treatment with these compounds. We therefore incubated primary rat hippocampal neurons overnight with KA and G (30 uM to 3 mM). Neuronal injury was determined by assaying lactate dehydrogenase (LDH) release. Dose-response curves showed different toxicity patterns for KA and G treatments. At lower concentrations KA was less toxic than G, but at concentrations higher than 300 uM, KA toxicity was 5 to 7 times of the control levels. G attained a toxicity level at most 2 to 3 times that of the baseline. Surprisingly, the toxicity of equimolar KA+G (300 =B5M + 300 =B5M) treatment was similar in magnitude to that induced by glutamate alone. Thus, the expected additive effects of toxic equimolar KA + G treatment was not obtained. One possible explanation for these results is the reported neuroprotective effect of activation of the metabotropic receptor by G; this action could mask the expected additive toxicity of KA+G treatments. A second possibility would involve the differential uptake of EAAs by astrocytes, which account for 10-15% of the cells in our culture. The role of both potential mechanisms will be investigated. (Supported by DAMD-17-93-V-3013).

1647

INHIBITION OF MUSCARINIC RECEPTOR BINDING BY PERVANADATE, ORTHOVANADATE, METAVANADATE AND AN ENDOGENOUS INHIBITOR FROM ALZHEIMER'S BRAIN. H. Venters Jr., M. Najarian, T. Ala and W.H. Frey II. Neurology, Ramsey Clinic, Health Partners, St. Paul, MN 55101.

Frey et al. have previously reported (Brain Res. **655**; 153-160, 1994) that an endogenous inhibitor (<3,500 daltons) of ^{3}H-QNB antagonist binding to the muscarinic receptor (mAChR) is elevated in brain tissue from patients with Alzheimer's disease (AD). Further research has suggested that this inhibition is irreversible and is mediated by the thiyl radical of glutathione which forms in the presence of the inhibitor. We now report the results of research on the effect of metavanadate (known to generate thiyl radicals from glutathione), orthovanadate, and pervanadate on ^{3}H-QNB binding to the mAChR. All three vanadium compounds inhibited binding in the presence of glutathione, with the order of decreasing potency and the concentration required for 50% inhibition (I_{50}) being: pervanadate (95 μM) > orthovanadate (132 μM) > metavanadate (452 μM). Omission of glutathione decreased the inhibition of the vanadium compounds from 2 to 6 fold. In contrast to our results with the endogenous AD inhibitor, preincubating the vanadium compounds with the mAChR in the presence of glutathione at 37°C for 1 hour markedly decreased the I_{50} values as follows: pervanadate (13 μM) > orthovanadate (46 μM) > metavanadate (118 μM). Similarities of the vanadium compounds with the endogenous AD inhibitor were also noted, with the inhibition of each being blocked by Trolox, EDTA and Mn^{2+}. Further studies are needed to identify the endogenous AD inhibitor. (Supported by Ramsey Foundation grant #N585).

1648

MECHANISM OF MASTOPARAN ACTION IN THE STIMULATION OF ACETYLCHOLINE RELEASE AND ITS INHIBITION BY BOTULINUM TOXIN A IN PC12 CELLS. P. Ray, M. Flink and W. Middleton. Walter Reed Army Institute of Research, Washington D. C. 20307.

We demonstrated that in PC12 cells, the mechanism of stimulated acetylcholine (ACh) release inhibition by botulinum toxin A (BoTx) was via interference of phospholipase A$_2$ (PLA$_2$) stimulated arachidonic acid (AA) release from cell membranes (Ray, *et al.*, J. Biol. Chem., 268:11057-11064, 1993). In this study, we further investigated the roles of PLA$_2$ and AA in ACh release and BoTx effect. Mastoparan (Mas), a PLA$_2$ activator, attenuated the BoTx effects. Mas increased intracellular free calcium concentration ([Ca^{2+}]$_i$) as well [^{3}H]AA and [^{3}H]ACh release in a concentration-dependent manner. The effects of Mas were enhanced synergistically by extracellular K$^+$. A specific Ca^{2+}-dependent cytosolic PLA$_2$ inhibitor, 7,7-Dimethyleicosadienoic acid (DEDA) abolished [^{3}H]AA and [^{3}H]ACh release due to Mas plus K$^+$, but the Ca^{2+}-independent specific PLA$_2$ inhibitor, (E)-6-(bromomethylene) tetrahydro-3-(*1*-napthalenyl)-2H-pyran-2-one (HELSS) had no effect. The effects of Mas plus K$^+$ were blocked by either EGTA or the N-type Ca^{2+} channel blocker ω-conotoxin, but not the L-type Ca^{2+} channel blocker nifedipine, indicating that these Mas effects were dependent on Ca^{2+} influx via the neuronal type voltage-sensitive Ca^{2+} channels. BoTx inhibition of [^{3}H]AA and [^{3}H]ACh release was fully prevented by Mas plus 80 mM K$^+$. These results indicate that Mas prevents the BoTx effects via Ca^{2+} influx, PLA$_2$ activation, and AA release; and provide additional support for our proposed role of AA in stimulated ACh release and its inhibition by BoTx.

1649

INCREASED NEUROTOXICITY FOLLOWING SIMULTANEOUS EXPOSURE TO PYRIDOSTIGMINE BROMIDE (PB), DEET, AND CHLORPYRIFOS. M.B. Abou-Donia, K.R. Wilmarth, Ali A. Abdel-Rahman, Karl F. Jensen, Frederick W. Oehme, and Thomas L. Kurt. Dept. of Pharmacol., Duke Univ. Med. Center, Durham, NC, 27710; USEPA, RTP, NC, 27711; Kansas State Univ., Manhattan, KS, 66506; Univ. of Texas, Southwestern Med. School, Dallas, TX, 75205.

The service personnel during the Persian Gulf War were exposed to pesticides such as the insect repellent, DEET (*N,N*-diethyl-*m*-toluamide) and the insecticide, chlorpyrifos (*O,O*-diethyl *O*-3,5,6-trichloropyridinyl phosphorothioate) and to PB (3-dimethylaminocarbonyloxy-*N*-methylpyridinium bromide) that was given as a prophylactic agent against possible nerve gas attacks. We determined the neurologic deficits produced by individual or coexposure of hens 5 days/week for 2 months to 5 mg PB /kg/day in water, po; 500 mg DEET /kg/day, neat, sc; and 10 mg chlorpyrifos kg/day in corn oil, sc. Binary treatments produced greater neurotoxicity than that caused by individual exposures. Neurotoxicity was further enhanced following coexposure to these chemicals. PB decreased plasma butyrylcholinesterase (BuChE) activity to 17% of control compared to 51% and 83% from chloryrifos and DEET, respectively. BuChE was inhibited further in combined treatment groups compared to individual dosed groups. In contrast, only chlorpyrifos alone or in combination with other compounds produced a significant inhibition of brain acetylcholinesterase activity. Brain neurotoxicity target esterase was significantly inhibited only in hens given chlorpyrifos with PB or DEET or with both chemicals. We hypothesized that test compounds compete for xenobiotic metabolizing enzymes in the liver and blood, and that carbamylation of peripheral aliesterases by PB increases the concentration of the lipophilic DEET and chlorpyrifos in circulation and their availability to the central nervous system. This increase in the effective concentrations of these chemicals in the nervous system could then reach levels equivalent to near-lethal doses of individual compounds associated with neurologic deficits. (Funding provided by the Perot Foundation.)

1650

THE THERAPEUTIC WINDOW OF 4-AMINOPYRIDINE, A COMPOUND EFFECTIVE AGAINST THE LETHAL EFFECTS OF SAXITOXIN. S.A. Keller, B.J. Benton, D. Spriggs, B.R. Capacio and F.-C.T. Chang (SPON: R. Ray). U.S. Army Med. Res. Inst. of Chem. Def., APG, MD 21010.

We have shown that saxitoxin (STX) induced lethality can be reversed by 4-aminopyridine (4-AP) if given at the time of respiratory arrest. The purpose of this study was to examine 4-AP's therapeutic window and to determine if its efficacy can be further enhanced when co-administered with STX. Three (3) groups of unanesthetized guinea pigs were used in this study. These animals were chronically instrumented for concurrent recordings of diaphragm EMG, skeletal muscle EMG, Lead II ECG and EEG. All animals were given a lethal dose of STX (5 μg/kg; im) which resulted in a progressive cardiorespiratory depression, and ultimately, respiratory arrest. Group 1 animals served as toxin controls. Group 2 animals received artificial ventilation at the point of respiratory arrest, followed by 4-AP (2 mg/kg, im). Group 3 animals were treated with 4-AP (2 mg/kg, im) immediately after STX and were ventilated at the time of apnea. Results from groups 2 and 3 animals showed that STX-induced cardiorespiratory failure could be restored to a level comparable to that of control. The diaphragm activities showed a slightly faster return in group 3 animals (1.13±0.21 min) as compared to group 2 animals (2.88±0.78 min). There was no difference, however, in the time it took for group 2 (42.1±8.5 min) and group 3 (39±7.1 min) animals to resume spontaneous breathing. In conclusion, we have shown that i) STX-induced lethality can be reversed by an experimental therapeutic modality which consists of artificial ventilation and 4-AP; and, ii) the therapeutic window of 4-AP is quite broad (from the time of intoxication to apnea); that is, administration of 4-AP at an earlier time point does not enhance the therapeutic effectiveness of 4-AP (as measured by the time taken for the return of spontaneous breathing).

2151

PAF INDUCES CYCLOOXYGENASE (COX-2) GENE EXPRESSION IN THE CORNEAL EPITHELIUM PARTIALLY BY A RECEPTOR-MEDIATED CALCIUM INFLUX.

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This study investigated the role and source of Ca^{2+} in the signaling of platelet-activating factor (PAF)-induced COX-2 gene expression in the corneal epithelium. Rabbit corneas were incubated in organ-culture in Hank's Ca^{2+}-free or minimum Eagle's medium, and the COX-2 mRNA expression in the epithelium was studied. Primary cultures of corneal epithelium were loaded with the fluorescent dye fluo-3, and changes of intracellular Ca^{2+} were analyzed by confocal microscopy. We found that PAF stimulated the expression of COX-2 mRNA that peaks at 4 hrs in the corneal epithelium incubated in MEM. The expression was inhibited by the PAF antagonist BN50730. When incubated in Ca^{2+}-free medium, there was a 40% inhibition of the induction. The Ca^{2+} ionophore A23187 caused a small but significant increase of COX-2 in the epithelium, which was abolished in Ca^{2+}-free medium. When added together, A23187 potentiated the effect of PAF. Confocal microscopic imaging showed that when incubated in Ca^{2+}-containing medium, PAF transiently increased intracellular Ca^{2+} which peaks between 30 and 60 seconds after adding PAF. Such effect of PAF was not seen when the cells were incubated with BN50730, when the cells were treated with vehicle, or when the cells were incubated in Ca^{2+}-free medium. In conclusion, Ca^{2+} is partially required in the induction of the COX-2 gene in the corneal epithelium. The source of such Ca^{2+} is likely to be extracellular, and its entry may be mediated via a PAF receptor. (NIH EY04928)

2153

PLATELET-ACTIVATING FACTOR MODULATES INTRACELLULAR CALCIUM DYNAMICS IN RAT HIPPOCAMPAL NEURONS.

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We have found using confocal microscopy and fluorescent calcium indicators, that intracellular calcium concentration ([Ca^{2+}]$_i$) oscillates spontaneously in rat hippocampal neurons *in vitro*. While addition of glutamate (GLU) to these hippocampal cultures consistently elicits distinct [Ca^{2+}]$_i$ changes ranging from transient, single spikes (100-500 nM GLU) to sustained increases (20-80 μM GLU), GLU does not appear to induce [Ca^{2+}]$_i$ oscillations. Here we have investigated the ability of the potent lipid mediator platelet activating factor (PAF) to affect [Ca^{2+}]$_i$ dynamics in hippocampal neurons. When 4 μM methylcarbamyl PAF (mcPAF) was added acutely to hippocampal neurons, the average [Ca^{2+}]$_i$ was increased slightly. The variance of fluorescence values after mcPAF additions was 8-fold higher than before additions, indicating an increase in oscillatory [Ca^{2+}]$_i$ dynamics induced by PAF. Neurons not spontaneously oscillating were observed to be induced to oscillate by PAF addition, and neurons spontaneously oscillating increased in oscillatory behavior upon PAF addition. In agreement with Bito *et al.* (Neuron, 9:285, 1992) we found that not all neurons responded to acute PAF application. Long-term treatment with PAF appeared to affect the majority of hippocampal cells. Overnight treatment with PAF (200-400 nM) and mcPAF (2-4 μM) reduced the neuronal [Ca^{2+}]$_i$ changes induced by GLU the next day when compared with cells pretreated with lysoPAF (2-4 μM) or vehicle alone. In two cases, the [Ca^{2+}]$_i$ increases in response to 500 nM GLU were completely inhibited by PAF pretreatment. Since PAF has been shown to enhance hippocampal excitatory synaptic transmission (Clark et al., Neuron 9:1211, 1992) we postulate that induction of [Ca^{2+}]$_i$ oscillations by PAF may be an early signal of GLU release, resulting in GLU receptor desensitization (Supported by DAMD-17-93-V-3013).

2155

RE-EVALUATION OF THE ROLE OF CaM KINASE II IN INSULIN SECRETION FROM PANCREATIC β-CELLS.

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Current evidence addressing the role of CaM kinase II in insulin secretion is conflicting. We have previously demonstrated that glucose activates the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM Kinase II) in a concentration-dependent manner that correlates with insulin secretion. Others have demonstrated that the CaM kinase II inhibitor, KN-62 (1 μM), failed to inhibit Ca^{2+}-induced insulin secretion from streptolysin O-permeabilized HIT cells, leading to the implication that this enzyme has no role in the secretory process. In this study, however, KN-62 at concentrations up to 100 μM did not inhibit CaM Kinase II activity in cellular extracts of βTC3 cells in the presence of exogenous calmodulin, and only modestly in the absence of the cofactor. In α-toxin-permeabilized βTC3 cells, Ca^{2+} induced the rapid activation of CaM kinase II in a concentration-dependent manner that was maintained for at least 30 min. This activation was not prevented by KN-62 (0-100 μM) negating the suggestion that CaM kinase II is not involved in Ca^{2+}-induced insulin secretion. The kinase inhibitor, K252a and a selective peptide inhibitor, [Ala^{286}] CaMK 281-302 strongly inhibited CaM kinase II activity in βTC3 cells and conditions have been established to permit the evaluation of the effects of these compounds on Ca^{2+}-induced insulin secretion. A more stringent correlation of the extent of inhibition of CaM Kinase II and insulin secretion by these compounds will permit a better assessment of the role of this enzyme in insulin secretion. (Supported by NIH 47925).

2152

POOL DEPLETION INDUCES A NOVEL CALCIUM INFLUX PATHWAY ACTIVATED BY CAFFEINE.

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Ca^{2+} influx through store-operated channels (SOCs) activated rapidly after Ca^{2+} pool depletion represents an important component of Ca^{2+} signals generated in cells. A new and distinct Ca^{2+} influx component activated by caffeine is induced in cells after Ca^{2+} pools are emptied using the intracellular Ca^{2+} pump inhibitors, thapsigargin (TG) or 2,5-di-*tert*- butylhydroquinone (DBHQ). Both blockers cause depletion of intracellular Ca^{2+} pools and cell growth arrest; upon refilling of pools, normal cell-cycle progression is resumed (Short, A.D., et al. PNAS 90, 4986-4990, 1993). Here, the Ca^{2+}-sensitive dye, fura-2, was used to study Ca^{2+} homeostasis in DDT_1MF-2 smooth muscle cells growth-arrested by TG- or DBHQ-treatment. In DDT_1MF-2 cells the SOC-mediated Ca^{2+} influx component after emptying Ca^{2+} pools is short- lived and appears to be rapidly deactivated. After treatment of DDT_1MF-2 cells with either 3 μM TG or 10 μM DBHQ, 10 mM caffeine induces a large transient influx of Ca^{2+} distinct from SOC- mediated Ca^{2+} entry. Caffeine-sensitive Ca^{2+} influx following DBHQ-treatment is activated more rapidly than that following TG-treatment. When caffeine is added to untreated DDT_1MF-2 cells no effect on cytosolic Ca^{2+} concentration is observed. The disappearance of caffeine-induced Ca^{2+} influx is also different for TG- and DBHQ-treated cells. In DBHQ-treated cells, bradykinin- sensitive Ca^{2+} pools quickly refill and cells become insensitive to caffeine immediately after DBHQ removal. In the case of TG-treated cells, reversal of TG-induced growth arrest with either high (20%) serum or 1-10 μM arachidonic acid, in addition to removal of TG, is required to allow agonist-sensitive Ca^{2+} pools to refill concomitantly with the disappearance of caffeine-induced Ca^{2+} influx. In summary, the results show that a Ca^{2+} influx pathway activated by caffeine is observed under conditions of growth arrest induced by either TG or DBHQ and appears to be directly correlated with depletion of intracellular Ca^{2+} pools. (NIH grants NS19304 and GM15407; NSF grant MCB 9307746).

2154

CALCIUM-STIMULATED PHOSPHORYLATION OF MAP-2 IN PANCREATIC β-CELLS IS MEDIATED BY CaM KINASE II.

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An elevation of intracellular Ca^{2+} is a critical signal in the initiation of insulin secretion from the pancreatic β-cell but the mechanism involved is not understood. Previously, we have demonstrated that the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is activated by glucose implicating this enzyme in the secretory process, but its cellular targets are unidentified. One of the best substrates of CaM kinase II *in vitro* that could function in secretory events, is the microtubule-associated protein, MAP-2. The current study represents an evaluation, *in situ*, of MAP-2 as a substrate of CaM kinase II using a permeabilized β-cell model. By immunoblot analysis, the presence of MAP-2 in the βTC3 cell was established. In α-toxin-permeabilized βTC3 cells, Ca^{2+} induced the concentration-dependent activation of CaM kinase II. In parallel and by immunoprecipitation, Ca^{2+} also induced the phosphorylation of MAP-2 that closely correlated with CaM Kinase II activation. Ca^{2+}-induced phosphorylation of MAP-2 was not inhibited by an inhibitor of protein kinase A (H89) at concentrations that prevented phosphorylation induced by forskolin. These data provide evidence that MAP-2 is phosphorylated by CaM kinase II in the pancreatic β-cell *in situ*, and that this event may provide an important link in the mediation of Ca^{2+}-dependent insulin secretion. (Supported by NIH grant DK-47925).

2156

OTHER EFFECTS OF ACETAZOLAMIDE IN RAT LIVER MITOCHONDRIA

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Preliminary studies using the carbonic anhydrase inhibitor acetazolamide showed a reduction in citrulline synthesis by intact guinea pig liver mitochondria (Arch. Biochem. Biophys. 1986. 251, 198-204) and it was concluded that this sulfonamide produces also an inhibition of urea synthesis in isolated guinea pig hepatocytes.

In this report we demonstrated that in rat liver mitochondria in the presence of different concentrations of acetazolamide, citrulline synthesis was decreased in a dose-response fashion obtaining the maximum inhibition (35%) with 300 μM; however, other effects were found. By using fluo-3 as a mitochondrial fluorescent calcium indicator, matrix free calcium ([Ca^{2+}]$_m$) was measured in a KCl-based buffer (pH 7.4) in the presence of EGTA. The effect of acetazolamide was a decrease (45%) of [Ca^{2+}]$_m$. When the same reaction was performed in the presence of 1 μM CaCl$_2$, mitochondrial matrix free calcium increased 3-4 fold (from 1.2 μM-3.6 μM) with 50-200 μM acetazolamide. The results obtained described another effect of the sulfonamide acetazolamide on mitochondrial matrix free calcium, which indicate that calcium ions could exert a physiological effect on citrulline synthesis.

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1476

HUMAN TYPE II PHOSPHOLIPASE A2 INDUCED Mac-1 EXPRESSION ON HUMAN NEUTROPHILS

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To investigate the effect of type II phospholipase A_2 (PLA_2-II) on neutrophil function, we assessed the Mac-1 expression on human neutrophils by flow cytometry after incubation of the cells with human PLA_2-II. PLA_2-II at a concentration of 10 μg/ml increased the Mac-1 expression by 150% compared with unstimulated cells at 30 min. Under thses conditions, PLA_2-II increased the exocytosis from secretory vesicles but not from either azurophilic, specific or gelatinase granules. The results suggest that PLA_2-II induces translocation of Mac-1 from the secretory vesicles to the plasma membrane. The Mac-1 induction mediated by PLA_2-II was inhibited by an anti-PLA_2-II antibody, which was able to inhibit the catalytic activity. However, the Mac-1 induction by PLA_2-II was not inhibited by either a 5-lipoxygenase, cyclooxygenase inhibitor or a PAF antagonist. Thus, we examined the effects of fatty acids and lysophospholipids on Mac-1 expression. Only arachidonic acid induced Mac-1 expression. These results imply that PLA_2-II induces Mac-1 expression on neutrophils via production of arachidonic acid.

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Regulation of Cytoplasmic Phospholipase A2(cPLA2) During Macrophage Differentiation and Lipid Loading

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Prostanoid formation by macrophage (MAC) and MAC-derived foam cells may have important autocrine and paracrine influences on cells of the atherosclerotic lesion. We examined the expression of cPLA2. a rate limiting enzyme in prostanoid synthesis, during MAC differentiation. Freshly isolated human monocytes had lower levels of cPLA2 mRNA and mass than differentiated MAC. mRNA levels doubled during 10 days in vitro differentiation and declined to 50% above baseline by day 15. cPLA2 mass paralleled changes in mRNA levels while enzymatic activity was regulated in a more complex manner. Removal of serum resulted in a rapid (2-4 hr) five fold rise in cPLA2 mRNA. protein, and enzymatic activity. MAC- conditioned medium blocked this increase suggesting the presence of a secreted autocrine regulator. THP-1 monocytic cells normally had higher basal levels of cPLA2 mRNA, protein, and enzymatic activity than fresh human monocytes. Terminal differentiation into MAC by 80nM phorbol ester resulted in loss of cPLA2 . Phorbol ester also downregulated expression of cPLA2 in primary MAC. Lipid loading of primary human MAC with acetylated LDL to produce foam cells, had little influence cPLA2 levels. The data suggest that lipid loading does not directly affect cPLA2 regulation but the differentiation stage of MAC may signficantly affect cPLA2 mediated prostanoid synthesis in the atherosclerotic lesion.

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MODULATION OF CALCIUM, NEUROTOXICITY AND ARACHIDONIC ACID RELEASE BY PHOSPHOLIPASE A TYPE II AND GLUTAMATE IN VITRO

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Secretory phospholipase A Type II ($sPLA_2$) may modulate neuronal function, under both physiological and pathological conditions. This 14 kDa enzyme, present in synaptic vesicles, is released by depolarization or neurotransmitter stimulation. Moreover, ischemia induces $sPLA_2$ gene expression in rat brain. We evaluated the effect of $sPLA_2$ from bee venom (BV) alone and with glutamate (GLU) on neurotoxicity through lactate dehydrogenase (LDH) release, intracellular free calcium concentration ($[Ca^{2+}]_i$), and $[^3H]$ arachidonic acid (AA) release on primary cultures of cortical neurons. At 0.01 μml, BV was not toxic, did not affect basal oscillations in ($[Ca^{2+}]_i$), but did induce [3H]AA release from phospholipids (PL). BV dose dependently (0.025-10 μg/ml) caused neurotoxicity, altered $[Ca^{2+}]_i$ dynamics, and stimulated [3H]AA release. GLU (80 μ M) toxicity was similar to 0.5 μ g/ml BV (100% above control). Concentrations of BV (0.01 to 0.05 μ g/ml), which resulted in a 40-80% increase in LDH release when combined with GLU (80 μ M), elicited synergy in neurotoxocity (2.5-fold higher LDH release than their individual LDH values) and [3H]AA (1.5-fold). MK-801 blocked the synergy but not the BV effects. In contrast to the sustained $[Ca^{2+}]_i$ response induced by GLU, BV dose-dependently (0.5-10 μ g/mL) induced a trancient increase in $[Ca^{2+}]_i$ followed by decreased basal oscillations and a significant fall in $[Ca^{2+}]_i$. These results indicate that calcium-independent toxicity may occur at low $sPLA_2$ concentrations and provide evidence for modulatory roles of $sPLA_2$s in neuronal signal transduction. Supported by DAMD-17-93-V-3013.

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Ceramide Inhibits PKC Activation of G-Protein-Dependent Phospholipase D *In Vitro*.

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We investigated ceramide inhibition of the activation of PLD in HL-60 cells and cell lysates. In intact HL-60 cells, phorbolmyristateacetate (PMA) activated PLD as measured by $[^3H]$palmitate-labeled phosphatidylcholine conversion to phosphatidylethanol (PEt) in the presence of 2% ethanol. C_6-Ceramide inhibited this PLD activation in intact HL-60 cells after 4 hr treatment and was maximally active at 10 μM. The activity was specific in that structural analogs were inactive. Although ceramide inhibited PMA-induced activation of PLD it did not inhibit translocation of PKC to the membrane. In a cell-free assay using exogenous dipalmitoylphosphatidyl$[^3H]$choline in a phosphatidylethanol-amine and phosphatidylinositol-4,5-bisphosphate liposome we confirmed that PLD is activated by a soluble G-protein. Ceramide had no effect on this activity under a variety of conditions. However, activation of PLD by GTPγS could be synergistically enhanced by the addition of PKC activators. This upstream effect was inhibited rapidly and specifically by ceramide (30 μM). Taken together, these data show that ceramide interferes specifically with PKC-mediated activation of PLD.

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INTERFACIAL ACTIVATION OF PHOSPHATIDYLINOSITOL-SPECIFIC PLC TOWARDS INOSITOL CYCLIC 1,2-MONOPHOSPHATE.

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Phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* catalyzes PI hydrolysis in discrete steps: (i) an intramolecular phosphotransferase reaction to form inositol cyclic 1,2-monophosphate (cIP); followed by (ii) a cyclic phosphodiesterase activity converting water-soluble cIP to inositol-1-phosphate. We have studied the phosphodiesterase activity of PI-PLC towards cIP with particular reference to the possibility of interfacial activation towards a substrate that does not partition in an interface. In comparison to the first step, PI-PLC had only modest activity towards cIP (K_m = 90 mM; V_{max} = 136 μmol min^{-1} mg^{-1}). The activity showed a cooperative dependence on cIP concentration (Hill coefficient of 2). Almost all detergents examined increased enzyme specific activity 2-fold (only at concentrations $\geq$ CMC) with little alteration of K_m. However, PC micelles or vesicles had a much more profound effect: K_m for cIP decreased to 29 mM with diC_7PC micelles added, V_{max} increased almost 7-fold, and the cooperativity in cIP binding was abolished. The phosphocholine headgroup was critical to this larger kinetic effect since other short-chain phospholipid micelles caused only the generic 2-fold increase observed with bile salts, Triton X-100, and other detergents. Monomeric PC led to a small activation that increased linearly with increasing monomer. ^1H NMR studies of partially deuterated PCs indicated that under these conditions the enzyme nucleated the binding of several PC molecules. This represents a novel and specific type of interfacial activation of a phospholipase toward a water soluble substrate. [Supported by GM 26762]

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EVIDENCE FOR THE EXISTENCE OF G-PROTEIN-DEPENDENT AND G-PROTEIN-INDEPENDENT ACTIVATION OF PHOSPHOLIPASE D IN LYMPHOCYTES.

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Previously we reported that tumor-promoting phorbol esters stimulate phospholipase D (PLD) independent of protein kinase C activation in bovine lymph node lymphocytes. (Cao et al., Biochem. Biophys. Res. Commun. 171, 955-962, 1990; IBID 217, 908-915, 1995). In the present study, we examined the effects of prostaglandins (PGs), E_2, $F_{2\alpha}$, D_2, and H_2 on PLD activity as measured by $[1\text{-}^{14}C]$ arachidonic acid incorporation into phosphatidylethanol (PEt) in bovine lymphocytes. All PGs stimulated the formation of PEt in a dose-dependent manner in the concentration range between 0.1 - 10μM. Prostaglandin E_2 had the maximum stimulatory effect in the order of $PGE_2 > PGF_{2\alpha} > PGH_2 > PGD_2$. The PG-stimulated formation of PEt was enhanced by staurosporine, a PKC inhibitor. When both were included, the effect of PGE_2 and 12-0-tetradecanoylphorbol-13-acetate (TPA) on the PLD activation was additive. Furthermore, NaF, a G-protein activator, stimulated the PEt formation and this stimulation was enhanced by staurosporine. Interestingly, the stimulatory effects of PGs and NaF were not additive; however, the formation of PEt by NaF and TPA was additive. These results suggest that, similar to TPA activation of PLD, PGs increase PLD activity independent of PKC and the stimulation by PGs and TPA in lymphocytes may involve both G-protein-dependent and G-protein-independent signaling pathways. (Supported through PHS grant numbers HL31245 and AI-06347.)

The inflammatory mediator platelet-activating factor and the inducible prostaglandin synthase (COX-2) gene in CNS diseases

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Platelet-activating factor (PAF, 1-0-alkyl-sn-2-acetyl-3-phosphocholine), the most potent biologically active lipid known, is involved in the injury/inflammatory response in many cells. In brain, PAF is rapidly produced at the onset of ischemia and seizures. PAF, the only bioactive phospholipid to have a cloned receptor, elicits actions also through an intracellular site. Physiologically, PAF modulates glutamate release, long-term potentiation (LTP) and memory formation. Furthermore, PAF is a transcriptional activator of COX-2. Interestingly, COX-2, as well as PAF, is involved in both synaptic plasticity (e.g., LTP) and in the injury/inflammatory response. Unlike in other cells, however, COX-2 is constitutively expressed in neurons at low levels.

Two CNS disease rat models were investigated to determine COX-2 is induced under pathological conditions and whether the antagonist that blocks PAF-induction of the COX-2 promoter inhibits, *in vivo*, COX-2 kainic acid-induced epileptogenesis. We find a sustained upregulation of COX-2 in hippocampus, several fold greater than another early response gene, *zif-268*. Pretreatment of animals with the intracellular PAF antagonist BN 50730 strongly attenuates COX-2 induction. In the second model, light-induced photoreceptor cell COX-2 protein accumulates preceding apoptotic nuclear changes and localizes to inner segments of photoreceptor cells. The PAF antagonist significantly reduces COX-2 light induction and apoptosis. Currently, we are testing the hypothesis that the overexpression of the PAF-COX-2 pathway leads to neuronal cell apoptosis.

Although it is often stated that the inflammatory response is not associated with apoptotic cell death, PAF and COX-2 appear to act as neuron injury messengers without reflecting classical "inflammatory" features to the entire tissue.

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